

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
21 November 2002 (21.11.2002)

PCT

(10) International Publication Number
WO 02/092002 A2

- (51) International Patent Classification⁷: A61K
- (21) International Application Number: PCT/US02/15012
- (22) International Filing Date: 9 May 2002 (09.05.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/290,481 11 May 2001 (11.05.2001) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/290,481 (CIP)
Filed on 11 May 2001 (11.05.2001)
- (71) Applicant (for all designated States except US): THE BURNHAM INSTITUTE [US/US]; 10901 North Torrey Pines Road, La Jolla, CA 92037 (US).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): HUANG, Shi [CN/US]; 5296 Timber Branch Way, San Diego, CA 92103 (US).
- (74) Agents: WEBSTER, Melanie, K. et al.; Campbell & Flores LLP, 7th Floor, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States (national): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

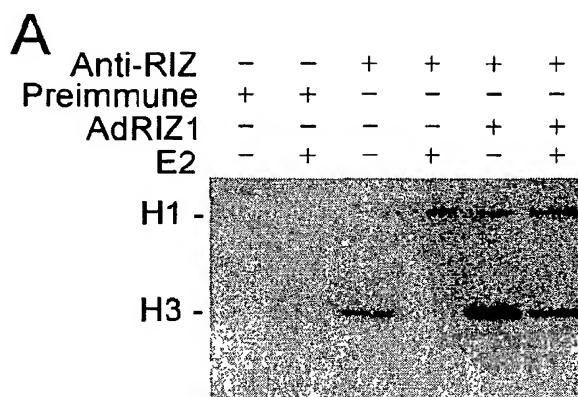
(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SCREENING, DIAGNOSTIC AND THERAPEUTIC METHODS RELATING TO RIZ



(57) **Abstract:** The invention provides a method of screening for a compound that modulates RIZ histone methyltransferase (HMT) activity, by contacting a RIZ or RIZ fragment having HMT activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. Also provided is a method of screening for a compound that modulates progesterone receptor (PR) activity, by providing a RIZ1 modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate PR activity. Further provided is a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy.

SCREENING, DIAGNOSTIC AND THERAPEUTIC
METHODS RELATING TO RIZ

This invention was made with United States
Government support under grant numbers CA76146 and
5 CA60988 awarded by the National Institutes of Health.
The U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields
10 of cancer biology and endocrinology and, more
specifically, to biological activities of RIZ
polypeptides.

BACKGROUND INFORMATION

Post-translational addition of methyl groups to
15 the amino-terminal tails of histone proteins is catalyzed
by a family of proteins known as histone
methyltransferases (HMTs). Histone site-specific
methylation is associated with a variety of fundamental
cellular processes, including transcriptional regulation,
20 epigenetic silencing and heterochromatin formation. Loss
of HMT function is expected to directly contribute to the
de-differentiation and genomic instability that are
characteristic of cancer.

Further implicating HMTs in cancer, it has been shown that a deficiency in dietary methionine or folate causes cancer. Dietary methionine and folate in turn regulate the cellular levels of S-adenosylmethionine (SAM), which is used as a methyl group donor by methyltransferases. Additionally, several independent lines of investigation have revealed that alterations in the methionine metabolic pathway, which can lead to a deficiency in SAM and/or an increase in the methyltransferase inhibitor S-adenosylhomocysteine (SAH), are associated with cancer.

Thus, there exists a need to identify proteins with histone methyltransferase activity, determine their substrates and regulators, and identify compounds that modulate their activity. In particular, there exists a need to identify histone methyltransferase activity within proteins already recognized to play critical roles in regulating cell proliferation. Compounds that modulate the HMT activity of such proteins are expected to be useful as therapeutics to regulate cell growth.

The steroid hormone progesterone is a critical component of the female reproductive cycle and is required to maintain pregnancy. The molecular target of progesterone is the intracellular progesterone receptor. Upon binding to progesterone, the progesterone receptor translocates to the nucleus where it binds as a transcription factor to DNA transcriptional elements present in progesterone-regulated genes.

Ligands to the progesterone receptor play an important role in female reproductive medicine and cancers. For example, progesterone and its synthetic analogs are useful in birth control formulations, in treating endometriosis and in maintaining pregnancy.

Antagonists to progesterone are useful in treating chronic disorders such as certain forms of hormone dependent cancer of the breast, ovaries and endometrium (the lining of the uterus) and in treating uterine
5 fibroids. Progesterone antagonists are also useful in combination with other drugs for terminanting early stage pregnancies.

Compounds that modulate progesterone receptor activity are thus expected to be useful in reproductive
10 applications and for treating malignant disorders. However, suitable high-throughput assays for identifying such compounds are currently lacking, in part due to a lack of understanding of physiologically relevant interactions of the progesterone receptor with cellular
15 molecules. Thus, there exists a need for improved methods to screen for compounds that modulate progesterone receptor activity.

The steroid hormone estrogen directly and indirectly promotes the proliferation of tissues
20 expressing estrogen receptors. Endocrine therapy to reduce the proliferative effects of estrogens, including therapy with selective estrogen receptor modulators (SERMs) such as tamoxifen, is currently the preferred first-line therapy in patients with estrogen receptor
25 positive (ER+) tumors. However, a significant portion of patients with ER+ tumors do not respond to endocrine therapy. In these patients, alternative therapies are warranted in order to improve the odds of survival. Currently, there is no satisfactory method of predicting
30 which ER+ tumors will respond to endocrine therapy.

Thus, there exists a need to identify molecules that correlate with loss of estrogen responsiveness in ER+ tumors, in order to more accurately determine which

individuals are more or less likely to respond to endocrine therapy. Using such a correlation, an individualized course of treatment with an improved likelihood of success can be chosen.

- 5 The present invention satisfies these needs, and provides related advantages as well.

SUMMARY OF THE INVENTION

 The invention provides a method of screening for a compound that modulates RIZ histone
10 methyltransferase activity. The method involves contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds, and determining histone methyltransferase activity of the contacted RIZ or RIZ fragment. A
15 compound that modulates RIZ histone methyltransferase activity is identified.

 The invention also provides a method of screening for a compound that modulates progesterone receptor activity. The method involves providing a RIZ1
20 modulatory compound, and determining the ability of the RIZ1 modulatory compound to modulate progesterone receptor activity. A compound that modulates progesterone receptor activity is thereby identified.

 Also provided is a method of identifying an
25 individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method involves determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood
30 of responding to endocrine therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the effect of RIZ1 on hormone responsive promoters. The indicated amounts of RIZ1 and various nuclear hormone receptor expression vectors were cotransfected with a reporter construct containing the appropriate hormone receptor response element into CV-1 cells. Cells were treated with or without the indicated hormone for 24 h and assayed for CAT activity. Data shown represent the means \pm SEM of three independent experiments.

Figure 1B shows that RIZ1 forms hormone-dependent protein complexes *in vivo* with ER α and progesterone receptor proteins. T47-D (for progesterone receptor) and MCF-7 (for ER) cells were infected with adenovirus expressing RIZ1. Cell extracts treated with or without hormones were prepared and immunoprecipitated (IP) with preimmune serum or monoclonal antibodies against progesterone receptor (PR) (detecting both PR-B and PR-A) or ER α , as indicated. The immunoprecipitated products were analyzed by immunoblot analysis using RIZ1, progesterone receptor or ER α monoclonal antibodies (2D7, 1A6, and sc-8005, respectively).

Figure 1C shows immunohistochemistry analysis of RIZ expression in target organs of female sex steroid hormones. Tissues from 7-week old virgin wild-type mice were analyzed for expression of RIZ and ER α . The sections show RIZ and ER α immunostaining in uterus (panels a and b), breast (c and d), and vaginal epithelium (e and f). Scale bar in a, c, and e also applies to b, d, and f respectively.

Figure 1D shows RT-PCR analysis of RIZ1 expression in target organs of female sex steroid

hormones. Total RNAs isolated from the indicated tissues of mice were used for RT-PCR analysis.

Figure 2 shows uterine and vaginal responses to female sex steroid hormones in RIZ1 mutant mice. A and B, Uterine wet weight was measured and the ratio of uterine weight to body weight calculated for the indicated number of mice and presented as mean \pm SEM. C. The uterine response to a decidual response was measured. After treatment with E2 and progesterone, and mechanical stimulation of the left uterine horn, the ratio of the weights of the stimulated (L) to the unstimulated (control; R) horn was calculated for the indicated number of mice and presented as mean \pm SEM. D and E. Vaginal responses. Vaginal tissues were collected and fixed for H & E staining. The thickness of the entire vaginal epithelium and the cornified layer were measured at 7 randomly selected areas at the maximum depth of the vaginal epithelium in each mouse and data are presented as mean \pm SEM for 7 mice in each group. F and G. Impaired regulation of uterine progesterone receptor expression by E2 in RIZ1 mutant mice. Uteri were collected and immunohistochemically analyzed for progesterone receptor expression. Estrogen treated uteri are shown in G (top panel: low magnification; bottom panel: high magnification). Randomly selected areas were photographed and more than 1000 cells from each compartment of the uteri of 4 mice in each group were counted, and data is presented as mean \pm SEM (G). Inserted scale bars apply for both genotypes.

Figure 3 shows mammapoiesis in RIZ1 deficient mice. Whole mounts of the fourth mammary gland of mice with the indicated genotypes were prepared and stained. A and B, Seven week old virgin mice. C and D, Mice pregnant for the first time. E and F, Higher

magnification of the ducts and alveolar structures of the mammary glands of the pregnant mice. G and H, Mammary glands from mice treated with hormone pellets containing P and E2 as described in methods. I and J, Higher magnification of the mammary ducts and alveolar structures from G and H, respectively. Scale bars that apply to both genotypes are inserted.

Figure 4 shows modulation by RIZ of E2-dependent transcription mediated by endogenous ER. MCF-7 cells (A) and CV-1 cells (B) were cotransfected with the reporter ERE-tk-CAT and either RIZ1 or RIZ2 expression plasmids. Cells were treated with or without E2 and assayed for CAT activity. Data shown represent the means \pm SEM of three independent experiments.

Figure 5 shows methylation of histones in vitro by the PR-domain, the PR-binding domain (PBD), and the combined action of the two domains. Bacteria-expressed GST fusion proteins of human RIZ1 were used for in vitro HMT reactions using histones as substrates. Top panel shows Coomassie blue staining of purified proteins and histones. Bottom panel shows fluorography indicating methylation of substrates. A. Methylation of H4 by GST-RIZ200 (consisting of RIZ1 residues 1-200) but not by GST protein alone, GST-RIZ161 (consisting of RIZ1 residues 1-161), and three GST-RIZ200 point mutant proteins as indicated. B. Methylation of H4 by GST-RIZ332 (consisting of RIZ1 residues 1-332). C. Enhanced methylation conferred by GST-RIZ322 plus GST-RIZPBD.

Figure 6 shows methylation of histones by full length RIZ1 protein expressed in mammalian cells. A. Nuclear extracts (500 μ g) prepared from U2OS cells infected with AdRIZ1 virus or AdNull control virus were

immunoprecipitated with preimmune serum or RIZ1 serum anti-KG7.1S. Immunoprecipitates were then assayed for HMT activity using free histones as substrates. The panel shows fluorography indicating methylation of

5 histone H1, H3 and H4. B. Enhanced methylation of lysine 9 on H3 by RIZ1 overexpression. Histones were extracted from U20S cells infected with AdRIZ1 or control virus and analyzed by western blot analysis using either antibody for methylated K9 on H3 or methylated K4 on H3.

10 C. Methylation of H3 N terminus peptide in vitro by RIZ1. *In vitro* methylation assays using immunoprecipitated RIZ1 as enzyme and the indicated N-terminal 1-20 residue peptide of H3 and acetylated H3 (K9/K14 Ac: acetylated on K9 and K14 residues) as

15 substrates. The methylated peptides were resolved by 16% Tris-Tricine SDS gel followed by fluorography. D. Automated sequencing of the H3 N-terminal peptide (residues 1-20) that had been methylated in vitro by full length RIZ1 purified by immunoprecipitation. The tritium

20 incorporation of individual amino acids identified at each successive round of microsequencing is shown.

Figure 7 shows regulation of RIZ1 methyltransferase activity by estrogen. A. Effects of estrogen on RIZ1 in MCF7 cells. Same amounts of nuclear

25 extracts (500 µg) from E2 treated or control MCF-7 cells, or from AdRIZ1 infected cells were immunoprecipitated with preimmune serum or RIZ1 serum anti-KG7.1S. Immunoprecipitates were then assayed for HMT activity using free histones as substrates. The panel shows

30 fluorography indicating methylation of histone H1 and H3. B. Effects of E2 and RIZ1 on H1 phosphorylation. MCF7 cells infected with control or AdRIZ1 virus were treated with E2 or vehicle for 48 hr. Histones were extracted after treatment and analyzed by western blot using

anti-phosph-H1 (top panel) or anti-H1 antibodies (bottom panel).

DETAILED DESCRIPTION OF THE INVENTION

The retinoblastoma-protein-interacting zinc
5 finger gene, or "RIZ" gene, was originally isolated in a
functional screen for proteins that bind to the Rb tumor
suppressor (Buyse et al., Proc. Natl. Acad. Sci. USA
92:4467-4471 (1995)). Two products of the RIZ gene are
produced, owing to alternative promoter usage. RIZ2 is
10 identical to RIZ1 except that it lacks the N-terminal 200
(rat) or 201 (human) amino acids (Liu et al., J. Biol.
Chem. 272:2984-2991 (1997)).

RIZ1 is considered to be a tumor suppressor
gene, as indicated by the following lines of evidence.
15 The RIZ gene maps to chromosome 1p36, one of the most
commonly deleted regions in human cancers. Gene
silencing of RIZ1, but not RIZ2, is common in many types
of human tumors - including breast cancer, liver cancer,
colon cancer, neuroblastoma, melanoma, lung cancer and
20 osteosarcoma (Chadwick et al., Proc. Natl. Acad. Sci. USA
97:2662-2667 (2000); He et al., Cancer Res. 58:4238-4244
(1998); Jiang et al., Int. J. Cancer 83:541-547 (1999)) -
and is associated with promoter DNA methylation (Du et
al., Cancer Res. 22:8094-8099 (2001)). RIZ1 missense
25 mutations (Steele-Perkins et al., Genes Dev. 15:2250-2262
(2001)) and frameshift mutations (Chadwick et al., supra
(2000); Piao et al., Cancer Res. 60:4701-47-4 (2000);
Sakurada et al., Genes, Chroms. Cancer 30:207-211 (2001))
are common in human tumors. RIZ1 expression induces
30 cell-cycle arrest, apoptosis induction and suppression of
xenograft tumors (Chadwick et al., supra 2000; He et al.,
supra (1998); Jiang et al., supra (1999); Jiang and
Huang, Cancer Res. 61:1796-1798 (2001)). Finally, mouse

gene knock-out models show that inactivation of RIZ1, while retaining normal RIZ2, causes tumor susceptibility (Steele-Perkins et al., supra (2001)). These studies suggest an important function for the N-terminus of RIZ1
5 in its tumor suppressor activity.

The N-terminus of RIZ1 contains a domain of about 130 residues called the "PR-domain," between about residues 30-160 of RIZ1. The PR-domain was initially identified as a region that showed homology between RIZ1
10 and the transcriptional repressor PRDI-BF1. This domain was later found to be structurally related to the SET-domain (Huang et al., J. Biol. Chem. 273:15933-15940 (1988)), which was recently shown to be a catalytic motif of lysine histone methyltransferases (HMTs) (Rea et al.,
15 Nature 406:593-599 (2000)). Despite the sequence similarity between PR and SET, the PR and SET domains are clearly distinct. First, identities among PR-domains or among SET domains are usually about 40%, whereas identities between PR and SET domains are typically only
20 about 20-30%. Second, SET domains are primarily found at the carboxyl termini of proteins, whereas PR-domains are mostly located at the amino termini. Prior to the instant disclosure, no enzymatic activity of a PR-domain had been described. Furthermore, histone
25 methyltransferase activity of the PR-domain of RIZ1, or of other domains within RIZ, had not been described.

As disclosed herein, both RIZ1 and RIZ2 proteins have histone methyltransferase activity.
30 Despite its actions as a tumor suppression, RIZ1 also acts as an estrogen receptor (ER) and progesterone receptor coactivator involved in regulating the normal proliferation and differentiation of estrogen- and progesterone-responsive tissues (see Example I). Because
35 of the dual role of RIZ as a growth suppressor and growth

promoter, compounds that modulate RIZ histone methyltransferase activity are expected to be useful in applications in which it desirable to positively or negatively modulate cell growth, including treatment of hyperproliferative disorders (e.g. neoplasia, hyperplasia, inflammatory conditions and the like) and treatment of hypoproliferative disorders (e.g. various disorders of hematopoiesis, wound healing and the like).

The invention thus provides a method of screening for a compound that modulates RIZ histone methyltransferase activity. The method is practiced by contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds, and assaying histone methyltransferase activity of the contacted RIZ or RIZ fragment. A compound that modulates histone methyltransferase activity of the RIZ or RIZ fragment is thereby identified.

As disclosed herein, HMT activity is present both in the PR-domain specific to RIZ1, and also in the C-terminal PR-binding domain (or PBD) common to RIZ1 and RIZ2. Accordingly, either RIZ1 or RIZ2, or fragments thereof that retain HMT activity, can be used in the invention methods.

The RIZ gene from several species has been cloned and characterized (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995); Liu et al., supra (1997)). Accordingly, a RIZ polypeptide from any species can be used in the invention screening methods. The human RIZ gene encodes the RIZ1 amino acid sequence set forth as SEQ ID NO:1 (GenBank Accession No. AAC50820; gi:9955379) and the RIZ2 amino acid sequence set forth as SEQ ID NO:2. The rat RIZ gene encodes the RIZ1 amino acid

sequence set forth in SEQ ID NO:3 (GenBank Accession No. AAA74468; gi:949996) and the RIZ2 amino acid sequence set forth as SEQ ID NO:4. The mouse RIZ gene has been partially sequenced (U.S. Patent No. 5,811,304).

5

Human and rat RIZ1 and RIZ2 polypeptides are highly homologous over their entire lengths (see, for example, U.S. Patent Nos. 6,323,335; 6,069,231; and 5,811,304). Because of this high degree of identity across two mammalian species, it is expected that other naturally occurring mammalian RIZ polypeptides, such as RIZ polypeptides from non-human primates, mouse, rabbit, bovine, porcine, ovine, canine or feline species, as well as naturally occurring RIZ polypeptides from other vertebrates, including fish, birds, reptiles and amphibians (e.g. *Xenopus*) will also exhibit a high degree of identity across their lengths with human and rat RIZ.

Using knowledge of the human or rat or RIZ-encoding nucleic acid sequences and polypeptides, those skilled in the art can readily clone RIZ-encoding genes from other species using conventional cDNA or expression library screening methods, or using the polymerase chain reaction (PCR). Additionally, using knowledge of the human or rat RIZ-encoding nucleic acid sequences and polypeptides, those skilled in the art can readily determine cDNA and coding sequences from other species from an analysis of ESTs and genomic sequences present in available databases. From these sequences, full-length RIZ1 or RIZ2 polypeptides, and fragments thereof with HMT activity, can be prepared for use in the screening methods disclosed herein.

A RIZ polypeptide useful in the methods of the invention can also have one or more minor modifications to the naturally occurring sequence, such as one or more

substitutions, additions or deletions. A modified RIZ polypeptide will generally retain at least 70%, 75%, 80%, 90%, 95%, 98% or higher identity with a native RIZ sequence. Such modifications can be advantageous, for example, in enhancing the stability, bioavailability, bioactivity or immunogenicity of the polypeptide, or to facilitate its purification.

Substitutions to a RIZ amino acid sequence can either be conservative or non-conservative. Conservative amino acid substitutions include, but are not limited to, substitution of an apolar amino acid with another apolar amino acid (such as replacement of leucine with an isoleucine, valine, alanine, proline, tryptophan, phenylalanine or methionine); substitution of a charged amino acid with a similarly charged amino acid (such as replacement of a glutamic acid with an aspartic acid, or replacement of an arginine with a lysine or histidine); substitution of an uncharged polar amino acid with another uncharged polar amino acid (such as replacement of a serine with a glycine, threonine, tyrosine, cysteine, asparagine or glutamine); or substitution of a residue with a different functional group with a residue of similar size and shape (such as replacement of a serine with an alanine; an arginine with a methionine; or a tyrosine with a phenylalanine).

Additions to a RIZ amino acid sequence include, but are not limited to, the addition of "tag" sequences, which are conveniently added at the N- or C- termini, after the signal peptide, or within extracellular or intracellular loops. Such tag sequence include, for example, epitope tags, histidine tags, glutathione-S-transferase (GST), fluorescent proteins (e.g. Enhanced Green Fluorescent Protein (EGFP)) and the like. Such additional sequences can be used, for example, to

facilitate expression, purification or characterization of the RIZ polypeptide.

Deletions to a RIZ amino acid sequence include, but are not limited to, deletion of residues at the N- or C- termini that are not critical for function. Deleted sequences can optionally be replaced by tag sequences or fusion sequences, as described previously.

Modifications to a RIZ amino acid sequence can be randomly generated, such as by random insertions, deletions or substitutions of nucleotides in a nucleic acid molecule encoding the polypeptide. Alternatively, modifications can be directed, such as by site-directed mutagenesis of a nucleic acid molecule encoding the polypeptide.

Guidance in modifying the sequence of a RIZ polypeptide while retaining biological activity can be provided by the alignment of the sequence of the RIZ orthologs from human and rat. It is well known in the art that evolutionarily conserved amino acid residues are more likely to be important for maintaining biological activity than less well-conserved residues. Thus, it would be expected that substituting a residue that is highly conserved among RIZ polypeptides across species with a non-conserved residue may be deleterious, whereas making the same substitution at a residue which varies among species would likely not have a significant effect on biological activity. An alignment of the rat and human RIZ1 amino acid sequences is presented in Figure 10 of U.S. Patent No. 5,811,304.

Computer programs well known in the art can also provide guidance in predicting which amino acid

residues can be modified without abolishing a structural or functional feature of a RIZ polypeptide.

As disclosed herein, RIZ histone methyltransferase activity is present in an N-terminal, PR-domain containing portion specific to RIZ1. Accordingly, an exemplary RIZ or RIZ fragment useful in the methods of the invention contains amino acids 1-200 of RIZ1, such as amino acids 1-332 of RIZ1. A further exemplary RIZ or RIZ fragment useful in the methods of the invention contains amino acids 1-520 of RIZ1. In contrast, a RIZ fragment consisting of residues 1-161 of human RIZ1 lacked HMT activity, as did a RIZ1 containing missense mutations in the PR-domain found in tumors, namely C106Y, I88V and A159V.

Additionally, as disclosed herein, RIZ histone methyltransferase activity is also present in a C-terminal PR-binding domain (PBD) containing portion common to RIZ1 and RIZ2 (Huang et al., J. Biol. Chem. 273:15933-15940 (1988)). Accordingly, an exemplary RIZ or RIZ fragment useful in the methods of the invention contains amino acids 1514-1680 of RIZ1, such as amino acids 1514 to the C-terminus of RIZ1.

The HMT activity of other RIZ polypeptides and fragments, including polypeptides and fragment with modified sequences as described above, can readily be determined by the assays described herein in order to select a RIZ or RIZ fragment having histone methyltransferase activity for use in the screening methods.

As used herein, the term "histone methyltransferase activity" or "HMT activity," with respect to a RIZ or RIZ fragment, refers to the ability

of the RIZ or RIZ fragment to catalyze the methylation of histones or histone peptides under suitable assay conditions. In contrast, under the same conditions, a control polypeptide, such as glutathione-S-transferase (GST), will not be able to catalyze the methylation of histones or histone peptides. HMT activity can be exhibited either in an *in vitro* assay with purified or partially purified RIZ, or in a cell-based assay. HMT activity includes HMT activity exhibited toward any histone, such as histone H1, H2A, H2B, H3 or H4.

Histones suitable for use as substrates in HMT activity assays can be obtained commercially (e.g. from Roche Molecular Biochemicals), prepared recombinantly based on known nucleic acid sequences, or extracted from cells using methods known in the art. Histone peptides suitable for use as substrates in HMT assays, including peptides with native sequences and peptides modified by acetylation on lysine residues, can be obtained commercially (e.g. from Upstate Biotech) or produced synthetically. Suitable histone peptides include, for example, H3 N-terminal peptides that include lys-9 (K9) (e.g. H3 amino acids 1-20) and H1 N-terminal peptides that include lys-25 (K25) (e.g. H1 amino acids 15-37 or 12-31). The methylation site on H4 is expected to be lys-20 (K20). Accordingly, a suitable histone H4 peptide can be an N-terminal peptide that includes lys-20.

For HMT assays, a RIZ or RIZ fragment of any desired sequence can conveniently be produced recombinantly, such as by expression of the encoding nucleic acid molecule in bacteria, yeast, insect or mammalian cells. The expressed polypeptide can then be isolated with anti-RIZ antibodies, or purified or partially purified by standard biochemical fractionation methods. Alternatively, to facilitate isolation, the RIZ

polypeptide can be expressed as a fusion with a tag sequence, such as glutathione-S-transferase (GST), a 6XHis tag or an epitope tag. Methods of producing and isolating tagged and untagged recombinant proteins are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Plainview, New York (2001); Ausubel et al. (Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)).

10 As an alternative to preparing a recombinant RIZ or RIZ fragment, an endogenous RIZ1 or RIZ2 can be purified or partially purified from a convenient cell or tissue source. A RIZ fragment can thus be prepared by enzymatic or chemical cleavage of the endogenous RIZ.
15 Alternatively, a RIZ fragment can be prepared by synthetic methods.

 HMT activity of a RIZ can be determined by methods known in the art. For example, the RIZ and a histone or histone peptide can be incubated with a
20 labeled methyl donor, such as
S-adenosyl-[methyl-¹⁴C]-L-methionine, or
S-adenosyl-[methyl-³H]-L-methionine, under suitable assay conditions. Transfer of the radiolabel to the histone or histone peptide can be detected, for example, by SDS-PAGE
25 electrophoresis and fluorography. Alternatively,
following the reaction the histone or histone peptides can be separated from the methyl donor by filtration, and the amount of radiolabel retained on the filter quantitated by scintillation counting. Other suitable
30 labels that can be attached to methyl donors, such as chromogenic and fluorescent labels, and methods of detecting transfer of these labels to histones and histone peptides, are known in the art.

Alternatively, HMT activity of a RIZ can be determined using an unlabeled methyl donor (e.g. S-adenosyl-L-methionine) and reagents that selectively recognize methylated histones or histone peptides. For example, after incubation of the RIZ, methyl donor and histones or histone peptides, under suitable assay conditions, methylated histones or histone peptides can be detected by immunoblotting or by an ELISA assay with antibodies specific for methylated histone epitopes. Suitable antibodies are described, for example, in Nakayama et al., Science 292:110-113 (2001), Noma et al., Science 293:1150-1155 (2001) and published U.S. Patent Application No. 20020039776, or can be prepared by methods known in the art (see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988)).

Instead of using antibodies, methylated histones can be detected using reagents that selectively bind methylated histones with high affinity. Such reagents are known in the art or can be determined by screening assays known in the art. An exemplary binding reagent is heterochromatin protein HP1, which binds histone H3 when methylated at lysine 9 (H3-K9). HP1, or a binding fragment thereof, can be labeled, and the HP1 or fragment bound to methylated H3-K9 detected. Alternatively, the HP1 or fragment need not be labeled, and can instead be detected using an anti-HP1 antibody in an ELISA assay.

Various low-throughput and high-throughput enzyme assay formats are known in the art and can be readily adapted for RIZ HMT assays. For high-throughput assays, the histone or histone peptide substrate can conveniently be immobilized on a solid support, such as a multiwell plate, slide or chip. Following the reaction,

the methylated product can be detected on the solid support by the methods described above. Alternatively, the HMT reaction can take place in solution, after which the histone or histone peptide can be immobilized on a solid support, and the methylated product detected. To facilitate such assays, the solid support can be coated with streptavidin and the histone labeled with biotin, or the solid support can be coated with anti-histone antibodies. The skilled person can determine suitable assay formats depending on the desired throughput capacity of the screen.

The invention screening method involves contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds and assaying histone methyltransferase activity of the contacted RIZ or RIZ fragment. A candidate compound useful in the methods of the invention can be a naturally occurring macromolecule, such as a peptide, nucleic acid, carbohydrate, lipid, or any combination thereof. A candidate compound alternatively can be a partially or completely synthetic derivative, analog or mimetic of such a macromolecule, or a small, synthetic molecule, such as an organic molecule prepared by combinatorial chemistry methods. A candidate compound can be detectably labeled or attached to a solid support, if desired, as appropriate in a particular assay.

Methods for producing large libraries of compounds, including simple or complex organic molecules, metal-containing compounds, carbohydrates, peptides, proteins, peptidomimetics, glycoproteins, lipoproteins, nucleic acids, antibodies, and the like, are well known in the art and are described, for example, in Huse, U.S. Patent No. 5,264,563; Francis et al., Curr. Opin. Chem. Biol. 2:422-428 (1998); Tietze et al., Curr. Biol.,

2:363-371 (1998); Sofia, Mol. Divers. 3:75-94 (1998);
Eichler et al., Med. Res. Rev. 15:481-496 (1995).

Libraries containing large numbers of natural and
synthetic compounds also can be obtained from commercial
5 sources.

The number of different candidate compounds to
screen in a particular assay can be determined by those
skilled in the art, and can be 2 or more, such as 5, 10,
15, 20, 50 or 100 or more different compounds. For
10 certain applications, such as when a library of random
compounds is to be screened, and for automated
procedures, it may be desirable to screen 10^3 or more
compounds, such as 10^5 or more compounds, including 10^7 or
more compounds. If desired, a plurality of candidate
15 compounds can be assayed in a pool, and the pool
repeatedly subdivided until a single compound with the
desired activity is identified. Candidate compounds can
be assayed simultaneously, in parallel, or sequentially.

The amount of candidate compound to use in a
20 reaction can be determined by the skilled person based on
the nature of the compound, the nature of the assay, and
the concentration of the reactants. If desired, a range
of doses of candidate compound can be tested.

Generally, the candidate compound will be
25 included in an HMT reaction together with the RIZ,
histone or histone peptide substrate, and methyl donor.
Optionally, the candidate compound and the RIZ can first
be incubated together, and then the other reactants
added. If desired, other components, such as different
30 RIZ polypeptides or RIZ fragments, can be included in the
reactions, and the effect of the candidate compound on
modulating RIZ HMT activity under such conditions

determined. The skilled person can determine suitable combinations of reactants and components.

For cell-based screening assays, a cell expressing a RIZ can be contacted with a candidate compound. Either the *in vivo* methylation of isolated histones can be determined following contacting, or the RIZ polypeptide can be isolated and its activity in methylating isolated histones or histone fragments assayed as described above. If desired, the ability of a candidate compound to modulate RIZ activity under physiologically relevant conditions can be determined in cell-based screening assays. For example, the ability of a candidate compound to modulate RIZ activity in the presence of estrogen or progesterone, in response to co-expressed polypeptides, or in tumor cells, can be determined.

Determining whether a candidate compound modulates RIZ HMT activity, either positively or negatively, generally requires comparison to a control. A control can be an identical reaction to the test reaction, except the control is not exposed to the candidate compound. The HMT activity of the control reaction can be assessed either before, after, or at the same time as the test reaction. A compound that "modulates" HMT activity is a compound that increases or decreases HMT activity, in comparison to a control, by at least 2-fold, such as at least 5-fold, 10-fold or more.

The results described in Example I suggest that the H3-K9 methylation activity of RIZ1 is linked with growth arrest, whereas its H1-K25 methylation activity is associated with cell proliferation. Accordingly, compounds that differentially regulate these two activities can be identified and used to promote or

inhibit cell growth, as appropriate for a particular application.

As disclosed herein, RIZ1, but not RIZ2, acts as a transcriptional coactivator of the nuclear hormone receptors for the female sex steroids estrogen (ER α) and progesterone. Furthermore, RIZ1 is required for normal growth and development of female target organs, such as the uterus, vagina, and mammary gland, in response to estrogen and progesterone. Therefore RIZ1, and particularly the PR domain of RIZ1, is a physiologically important regulator of progesterone and estrogen receptor activity. Compounds that modulate RIZ1 activity are thus likely to also be able to modulate progesterone receptor and/or ER α activity. Such compounds can be used as therapeutics to prevent, ameliorate or treat conditions that are benefitted by modulated female sex steroid receptor activity, including reproductive conditions and cancer.

In one embodiment, the invention provides a method of screening for a compound that modulates progesterone receptor activity. The method is practiced by providing a RIZ1 modulatory compound, and determining the ability of the compound to also modulate progesterone receptor activity.

As used herein, the term "RIZ1 modulatory compound" refers to a compound that affects a RIZ1 biological activity, such as a binding interaction with a cellular molecule or an enzymatic activity (e.g. histone methyltransferase activity). A RIZ1 modulatory compound can thus be, for example, a compound that selectively binds RIZ1, a compound that modulates (by either increasing or decreasing) an interaction between RIZ1 and a binding partner, a compound that modulates a functional

activity of RIZ1, or a compound with several of these effects.

Compounds that selectively bind RIZ1 are known in the art or can be identified by available screening
5 methods. For example, compounds that selectively bind RIZ1 include RIZ1-specific antibodies (described, for example, in U.S. Patent No. 5,811,304) and RIZ1 binding partners. As used herein, the term "RIZ1 binding partner" is intended to refer to a cellular molecule that
10 normally binds with high affinity to a RIZ1, such as cellular proteins, nucleic acid molecules, enzymatic substrates and cofactors, the RIZ1-binding domains from these molecules, and RIZ1-binding variants and analogs of these molecules.

15 A "variant" of a RIZ1-binding partner has substantially the same amino acid or nucleotide sequence as the native molecule (e.g. at least 70%, 75%, 80%, 90%, 95%, 98% or higher identity), and retains substantially the same binding activity as the native molecule. In the
20 case of a polypeptide, the polypeptide can have one or more conservative or non-conservative amino acid substitutions, additions or deletions compared to the naturally occurring sequence. Methods of preparing and assaying the binding activity of variants of RIZ1-binding
25 partners are well known in the art.

An "analog" of a RIZ1-binding partner has substantially the same core chemical structure as the native molecule, and retain substantially the same binding activity as the native molecule. Methods of
30 preparing and assaying the binding activity of analogs of RIZ1-binding partners are well known in the art.

An exemplary cellular protein that binds RIZ1 is the retinoblastoma protein (Rb). U.S. Patent No. 5,811,304 describes the specific binding of RIZ1 to full-length Rb and to a C-terminal 56 kDa fragment of Rb that contains the Rb binding pocket (see also Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995)). Other cellular proteins that selectively bind RIZ1 include the estrogen receptor (ER α) (see Abbondanza et al., Proc. Natl. Acad. Sci. USA 97:3130-3135 (2000) and Example I) and progesterone receptor (PR) (see Example I). Additionally, GATA-3 (Shapiro et al., Gene 163:329-330 (1995)), p53, SRC1 and p300 are exemplary cellular proteins that selectively bind RIZ1.

Nucleic acid molecules that selectively bind RIZ1 are described, for example, in U.S. Patent No. 5,811,304. Exemplary nucleic acid molecules to which RIZ1 selectively binds, through its zinc finger DNA binding motifs, contain GC-rich or Sp-1-binding elements.

Exemplary enzymatic substrates and cofactors that selectively bind RIZ1 include GTP (see U.S. Patent No. 5,811,304), zinc ions (which associate with the zinc finger motifs) and S-adenosyl-methionine (SAM).

SAM acts as a substrate and methyl group donor for RIZ1 methyltransferase activity. Analogs of SAM include S-adenosyl-L or D-homocysteine (SAH), and adenosyl-L-ethionine. Other binding compounds can include, for example, A-adenosyl-gamma-thio-alpha-ketobutyrate, S-adenosyl-L-homocysteine sulfoxide, methylothioadenosine (MTA), L-homocysteine, L-homoserine, Adenosine, Adenine, ATP, CAMP and methionine.

Therefore, any of the above compounds, or variants, analogs, agonists and antagonists of these

compounds, can be assayed for their ability to modulate progesterone receptor activity by the methods of the invention.

Compounds that modulate the interaction between
5 RIZ1 and a binding partner are also known in the art or can be identified by available screening methods. For example, U.S. Patent No. 5,811,304 describes a 17 amino acid Rb binding peptide (101-118: T-pep) from the SV40 large T antigen as a compound that interferes with the
10 interaction between RIZ1 and Rb. Other molecules that bind to a RIZ1 binding partner, or include a binding domain from a RIZ1 binding partner, are expected to be compounds that modulate the interaction between RIZ1 and a binding partner. Other compounds that can modulate the
15 interaction between RIZ1 and a binding partner can contain a RIZ1 binding domain, and thus compete with full-length RIZ1 for the binding partner. RIZ binding domains include, for example, the LXXLL steroid receptor binding motif described herein, as well as the
20 E1A-related region; the leucine-zipper; conserved regions 1 and 2; common epitope 1; zinc fingers; the GTPase domain; the SH3 domain; the SH3-binding domain; and the PMT (PR) domain, each of which is described in U.S. Patent No. 5,811,304.

25 Other compounds that selectively bind RIZ1, or that modulate the interaction between RIZ1 and a binding partner, can be identified by either manual or high-throughput screening assays, starting from a library of known or unknown compounds, as described above.

30 A binding assay can use a detectably labeled candidate compound and an unlabeled RIZ1 (and optionally an unlabeled binding partner). Alternatively, a binding assay can use an unlabeled candidate compound or binding

partner and a labeled RIZ1. Other appropriate combinations of labeled and unlabeled molecules can be determined by the skilled person depending on the assay format.

5 A variety of competitive and non-competitive binding assay formats for determining binding between molecules are known in the art. These assays include both solution-based methods and solid phase methods (e.g. molecules bound to plates, chips, affinity columns and
10 the like). Binding assays are amenable to either manual or high-throughput automated screening of compounds. Two exemplary binding assays are set forth in Example IIA and IIB.

 Detectable labels can include, for example, a
15 radioisotope, fluorochrome, ferromagnetic substance, or luminescent substance. Exemplary radiolabels useful for labeling compounds include ^{125}I , ^{14}C and ^3H . Methods of detectably labeling organic molecules, either by incorporating labeled amino acids into the compound
20 during synthesis, or by derivatizing the compound after synthesis, are known in the art.

 RIZ1 functional activities are described herein or are known in the art. Exemplary activities include, for example, transcriptional activation (see, for
25 example, U.S. Patent No. 5,811,304), transcriptional repression (see, for example, Xie et al., J. Biol. Chem. 272:26360-26366 (1997)), histone methyltransferase activity (see Example I) and hormone receptor coactivation (see Examples I and II).

30 Suitable assays for identifying compounds that modulate RIZ1 transcriptional activation, repression and coactivation function can be determined by the skilled

person. Such assays are generally based on co-expression of RIZ1 and an appropriate promoter-linked reporter gene in a cell, under conditions where a certain amount of transcription occurs, contacting the cell with the
5 candidate compound, and determining whether there is a change (i.e. either an increase or decrease) in transcriptional activity. Transcription based assays are well known in the art, and readily amenable to high-throughput screening assays. Methyltransferase activity
10 assays have been described above.

A compound that is identified as a RIZ1 modulatory compound by any of the above methods is then tested, in either an *in vitro* or *in vivo* assay, to determine whether it also modulates progesterone receptor
15 activity. A compound that "modulates" progesterone receptor activity is intended to refer to a compound that either increases or decreases a PR biological activity.

The progesterone receptor is normally present in an inactive form in the cytoplasm; where it interacts
20 with molecular chaperones, immunophilins, and heat shock proteins. The active PR binds progesterone and translocates to the nucleus where it binds as a transcription factor to canonical DNA transcriptional elements present in progesterone-regulated genes. As
25 described herein, RIZ1 coactivates the transcriptional activity of PR. A compound that modulates progesterone receptor (PR) activity can thus be, for example, a compound that alters the interaction between PR and the molecules that normally hold it in an inactive form;
30 alters the interaction between PR and progesterone; alters the interaction between RIZ1 and PR; or directly increases or decreases the transcriptional activity or specificity of PR toward PR-regulated genes.

Competitive and non-competitive binding assays have been described above with respect to RIZ1, and can be applied to identifying compounds that modulate PR interactions with progesterone, regulatory molecules or RIZ1. Assays that measure PR transcriptional activity can also be used to identify compounds that modulate PR activity. Conveniently, the progesterone response element (PRE) can be operatively linked to a reporter gene (e.g. luciferase, green fluorescent protein, beta-galactosidase and the like) and expressed in a cell, and an increase or decrease in expression of the reporter gene in response to a compound determined. Such an assay is described in Example I and IIC. Alternatively, a gene that is naturally induced by PR can be used in such assays, such as casein, and the mRNA or protein product detected.

Transcription-based assays, reporter genes, and methods for detecting gene expression, and methods of adapting these assays for high-throughput screening are well known in the art and described, for example, in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (2001).

Other assays for compounds that modulate progesterone receptor activity include *in vivo* assays that report PR function, such as the assays for uterine, vaginal and mammary development described in Example I and IID. A change in development of these organs in response to treatment of the animal with a particular compound is consistent with the compound having an effect on PR activity.

Once identified, compounds that modulate progesterone receptor activity can be formulated as pharmaceutical compositions and used to prevent or treat

reproductive and endocrinological disorders and cancers. Such disorders can include, for example: infertility, tubal disease, ovulatory defects, endometriosis, disruptions of the estrous cycle, disruptions of the menstrual cycle, polycystic ovary syndrome, ovarian hyperstimulation syndrome, endometrial and ovarian tumors, autoimmune disorders, ectopic pregnancy, teratogenesis, cancer of the breast, fibrocystic breast disease, galactorrhea, disruptions of spermatogenesis, abnormal sperm physiology, cancer of the testis, cancer of the prostate, benign prostatic hyperplasia, prostatitis, carcinoma of the male breast and gynecomastia. Other conditions in which increasing or decreasing progesterone receptor activity would be beneficial are known in the art or can be determined by the skilled person. The invention thus provides for the administration of a pharmaceutical composition containing a compound that modulates progesterone receptor activity, to prevent or ameliorate any of the disorders and conditions described above.

The pharmaceutical compositions of the invention, which include compounds that modulate RIZ1 HMT activity, and compounds that modulate progesterone receptor activity, may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of

routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, 5 sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of 10 the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

15 Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as 20 tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with 25 solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, 30 sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and

tragacanth; and proteins such as gelatin and collagen.
If desired, disintegrating or solubilizing agents may be
added, such as the cross-linked polyvinyl pyrrolidone,
agar, alginic acid, or a salt thereof, such as sodium
5 alginate.

Pills, capsules, tablets and the like may
further contain suitable coatings, such as concentrated
sugar solutions, which may also contain gum arabic, talc,
polyvinylpyrrolidone, carbopol gel, polyethylene glycol,
10 and/or titanium dioxide, lacquer solutions, and suitable
organic solvents or solvent mixtures. Dyestuffs or
pigments may be added to the pills or coatings for
product identification or to characterize the quantity of
active compound, i.e., dosage.

15 Pharmaceutical preparations which can be used
orally include push-fit capsules made of gelatin, as well
as soft, sealed capsules made of gelatin and a coating,
such as glycerol or sorbitol. Push-fit capsules can
contain active ingredients mixed with a filler or
20 binders, such as lactose or starches, lubricants, such as
talc or magnesium stearate, and, optionally, stabilizers.
In soft capsules, the active compounds may be dissolved
or suspended in suitable liquids, such as fatty oils,
liquid, or liquid polyethylene glycol with or without
25 stabilizers.

Pharmaceutical formulations suitable for
parenteral administration may be formulated in aqueous
solutions, preferably in physiologically compatible
buffers such as Hanks' solution, Ringer's solution, or
30 physiologically buffered saline. Aqueous injection
suspensions may contain substances which increase the
viscosity of the suspension, such as sodium carboxymethyl
cellulose, sorbitol, or dextran. Additionally,

suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling can include amount, frequency, and method
5 of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an
10 effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, or in animal models, usually mice, rats, rabbits,
15 dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

20 A therapeutically effective dose refers to that amount of active ingredient which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the
25 dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit
30 large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably

within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 μ g to 100 mg, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art.

As disclosed herein, RIZ1 is an estrogen receptor coactivator. RIZ1 silencing is significantly more common in ER-negative breast cancers compared with ER-positive breast cancers, and appears to be implicated in the acquisition of estrogen resistance, since nearly half of ER+ cancer tissues examined were determined to be RIZ1 expression-negative.

The action of estrogen is mediated via interaction with a specific receptor (ER) that initiates a series of downstream events, leading to the modulation of hormone-responsive genes and cell proliferation.

- 5 Endocrine therapy, including both surgical and medical therapies to reduce estrogen action, is the preferred first-line therapy in patients with ER+ cancers.

However, a large percentage of patients with ER-positive tumors do not respond to endocrine therapy.

- 10 The determination that RIZ1 inactivation is highly associated with decreased estrogen responsiveness in breast cancer cells allows RIZ1 status to be used to predict whether an individual with an ER+ tumor is likely to respond to endocrine therapy.

- 15 Based on information regarding RIZ1 status, a clinician can determine a suitable treatment for the particular individual that is more likely to be effective in prolonging disease-free survival and/or reducing mortality, while avoiding exposing the individual to
20 unnecessary treatments with potential side effects. For an individual determined by the invention method to have a normal likelihood of responding to endocrine therapy, endocrine therapy is appropriate. However, for an individual determined by the invention method to have a
25 reduced likelihood of responding to endocrine therapy, an alternative treatment can be chosen from among the available treatment options. Alternatively, for such an individual, endocrine therapy can be combined with an additional treatment.

- 30 The nature of an alternative or additional treatment for such an individual can be chosen by the clinician depending on the type and stage of tumor, extent of metastasis, overall health of the individual

and other concurrent treatments. For example, in an individual determined to have a reduced likelihood of responding to endocrine therapy, chemotherapy, surgery or radiation can be combined with, or used as an alternative to, endocrine therapy.

Accordingly, the invention provides a method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy. The method is practiced by determining the RIZ1 status of the tumor, wherein an abnormal RIZ1 status identifies the individual as an individual with a reduced likelihood of responding to endocrine therapy. A normal RIZ1 status identifies the individual as an individual with a normal likelihood of responding to endocrine therapy.

As used herein, the term "endocrine therapy" refers to a therapy that reduces the proliferative potential of estrogen. Endocrine therapy can be either surgical or medical, and affect any aspect of estrogen action, including estrogen production and estrogen receptor signaling.

Methods of ablating estrogen production include surgical removal or irradiation of the ovaries, which are the primary source of estrogen in premenopausal women. Ablation of estrogen production can also be achieved pharmacologically by using luteinizing hormone-releasing hormone or gonadotropin-releasing hormone agonists.

Selective estrogen receptor modulators (SERMs) refer to compounds that blunt or block the effects of an estrogen agonist, such as 17 β -estradiol, when administered concomitantly in a test system. SERMs used in cancer therapy, although commonly known as "anti-

estrogens," generally exhibit a combination of anti-estrogenic and estrogenic effects. The antagonist effect of tamoxifen, the most widely used SERM, is a result of blocking the transmission of E2-ER-initiated signals for cell proliferation. Other SERMs include 4-hydroxytamoxifen and related triphenylene antiestrogens; clomiphene; and non-uterotrophic antiestrogens, such as raloxifene, droloxifene, idoxifene, nafoxidine, toremifene, TAT-59, levomeloxifene, LY-353381, CP-336156, MDL-103323, EM-800, ERA-923, ICI-182,780 and the like. Other SERMs are known in the art or can be determined.

Aromatase inhibitors are also used in endocrine therapy for ER+ cancers in postmenopausal women. Aromatase catalyzes the final step in the synthesis of the estrogens estrone and estradiol from androgens and, therefore, inhibitors of aromatase reduce estrogen production. Exemplary aromatase inhibitors include aminoglutethimide, megestrol acetate, anastrozole, letrozole and exemestane.

As used herein, the term "estrogen receptor positive (ER+) tumor" refers to a tumor that expresses estrogen receptors above an art-recognized threshold level. A number of assays are routinely used in the art to categorize a tumor as ER+ or ER-. Such assays include, for example, ligand binding assays (LBA) and immunohistochemistry (IHC). Conveniently, immunohistochemistry can allow for simultaneous determination of ER status and RIZ1 status of a tumor, as described herein. Generally, an ER level of greater than 3fmol/mg cytosolic protein by an LBA is considered ER+, whereas greater than 1% positive cells by an IHC assay is considered ER+ (Harvey et al., J. Clin. Oncol. 17:1474-1481 (1999); Elledge et al., Int. J. Cancer (Pred.

Oncol.) 89:111-117 (2000)). A cutoff of 10% positive cells was considered ER+ in the immunohistochemical assays described in Example I. However, the results would not have changed significantly using a 1% cutoff.

5 As used herein, the term "tumor" refers to a malignant neoplasm. A tumor can be either a primary lesion or a metastatic lesion. Tumors that have been shown to express estrogen receptors include breast carcinoma, endometrial carcinoma, prostate carcinoma,
10 ovarian carcinoma, renal carcinoma, melanoma, colorectal tumors, desmoid tumors, pancreatic carcinoma, and pituitary tumors. The methods of the invention are applicable to ER+ tumors of any of these types, as well as other tumors determined to be ER+. The methods of the
15 invention are applicable to ER+ cancers at any clinical stage, including localized cancers, cancers with regional spread and cancers with distant spread.

 Additionally, the method can be practiced with respect to individuals whose tissues upon biopsy are
20 determined not to be malignant, including tissues that are determined to be normal or precancerous. An abnormal RIZ1 status in such ER+ tissues is predictive of an increased likelihood of the individual developing a tumor in that tissue. In particular, in women with low amounts
25 of circulating estrogen, including post-menopausal women and women whose ovaries have been removed, an abnormal RIZ1 status in ER+ tissues is predictive of an increased likelihood of the individual developing a tumor in that tissue. For such individuals, prophylactic treatment,
30 other than endocrine therapy, can be warranted. Suitable prophylactic treatments, including lifestyle changes (e.g. diet and exercise) and drug treatments, can be determined by those skilled in the art.

As used herein, the term "status" with respect to RIZ1 refers to a measurable property that is correlated with RIZ1 polypeptide function in the tumor. The measurable property that is determined can be a
5 property of the RIZ1 polypeptide or of the encoding RIZ1 gene or mRNA.

An exemplary measurable property correlated with RIZ1 polypeptide function in the tumor in the tumor is the amount of the RIZ1 gene, mRNA or polypeptide in
10 the tumor. An "abnormal RIZ1 status" in this case can thus be a decreased amount, in the tumor sample, of the RIZ1 gene, mRNA or polypeptide. Generally, a decreased amount of the RIZ1 gene, mRNA or polypeptide in the tumor refers to at least an 80% reduction, such as a 90%
15 reduction, in the amount of the RIZ1 gene, mRNA or polypeptide in the tumor sample, relative to the amount in a control sample. The skilled person can determine an appropriate control for a particular assay, such as nearby normal tissue from the same individual, or a tumor
20 sample previously identified as having a normal amount of the RIZ1 gene, mRNA or polypeptide.

A further exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is the structural integrity of the RIZ1 gene. An "abnormal
25 RIZ1 status" in this case can thus be a mutation in the normal nucleotide sequence of the RIZ1 gene in the tumor sample. Such a mutation can be determined directly by sequencing all or selected portions of the RIZ1 gene or mRNA. A mutation in the RIZ1 gene sequence can also be
30 inferred indirectly, such as by detecting an alteration in the normal size or abundance of the RIZ1 mRNA, an alteration in the normal ratio of RIZ2/RIZ1, or by detecting an alteration in the normal size, stability or localization of the RIZ1 polypeptide product. Mutations

that can affect the ability of the RIZ1 polypeptide to be expressed or function normally include, for example, genomic deletions, frameshift mutations (Chadwick et al., Proc. Natl. Acad. Sci. USA 97:2662-2667 (2000); Piao et al., Cancer Res. 60:4701-4704 (2000); Sakurada et al., Genes, Chroms. Cancer 30:207-211 (2001)), missense mutations, nonsense mutations, and mutations in regulatory regions (U.S. Patent No. 5,811,304).

Methods for determining the amount or structural integrity of a particular gene, mRNA or polypeptide in a sample are well known in the art and described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Plainview, New York (2001); Ausubel et al. (Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)). The particular method for a given application can be chosen by the skilled person. Exemplary methods include, for example, DNA sequencing, SSCP, Southern blotting, PCR, Northern blotting, RT-PCR, RNase protection, *in situ* hybridization, immunohistochemistry, immunoblotting and immunoprecipitation. Reagents suitable for detecting RIZ1 nucleic acid molecules in these methods, such as hybridization probes and PCR primers, are known in the art and described, for example, in He et al., Cancer Res. 4238-4244 (1998) and in the Example herein. Reagents suitable for detecting RIZ1 polypeptide in these methods, such as the KG7.1S and 2D7 antibodies, are also known in the art and described, for example, in Buyse et al. Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995).

Another exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is the extent of methylation of the RIZ1 gene promoter. As described in Du et al., Cancer Res. 61:8094-8099 (2001),

partial or complete methylation of the 35 CpGs in the RIZ1 promoter is strongly correlated with reduced RIZ1 mRNA expression, which in turn results in reduced RIZ1 polypeptide abundance. An "abnormal RIZ1 status" in this case can thus be partial or complete RIZ1 promoter CpG methylation.

RIZ1 promoter methylation can be determined, for example, by the methods described in Du et al., supra (2001). Briefly, one method involves treating genomic DNA from a sample with bisulfite, amplifying the RIZ1 promoter DNA using polymerase chain reaction (PCR), followed by cloning and sequencing the amplified product. Bisulfite treatment converts unmethylated cytosines to uridine, whereas methylated cytosines are resistant to conversion. An exemplary primer set suitable for RIZ1 promoter amplification by this method is 5'-GGTTGGGTGGTGGTTATTGGG-3' (SEQ ID NO:5) and 5'-CAAAAACCGCCCTGCGCCACTCCTTACC-3' (SEQ ID NO:6). An alternative method involves treating genomic DNA from a sample with bisulfite and amplifying the DNA using methylation-specific polymerase chain reaction (MSP) with RIZ1 specific primers. An exemplary primer set for selectively amplifying methylated RIZ1 promoter DNA is 5'-GTGGTGGTTATTGGGCGACGGC-3' (SEQ ID NO:7) and 5'-GCTATTTGCGCCGACCCGACG-3' (SEQ ID NO:8), whereas an exemplary primer set for selectively amplifying unmethylated RIZ1 promoter DNA is 5'-TGGTGGTTATTGGGTGATGGT-3' (SEQ ID NO:9) and 5'-ACTATTTACCAACCCCAAGA-3' (SEQ ID NO:10).

Another exemplary measurable property correlated with RIZ1 polypeptide function in the tumor is histone methyltransferase activity of the RIZ1 polypeptide. An "abnormal RIZ1 status" in this case can thus be altered H1-K25 methylation activity, altered

H3-K9 methylation activity, or an alteration in the normal RIZ activity toward another identified histone methylation site. Such an alteration can be either increased or decreased HMT activity, relative to a normal control. Histone methyltransferase assays have been described above, and can be adapted by the skilled person for determining histone methyltransferase activity of a RIZ1 polypeptide in a tumor sample.

The skilled person can determine other measurable properties correlated with RIZ1 polypeptide function in the tumor, which can be used in the invention method to determine RIZ1 status. If by any of these criteria it is determined that the RIZ1 polypeptide would be unlikely to function normally in the tumor, whether because of altered amount, structure or activity, the tumor is designated as having an "abnormal RIZ1 status," and the individual identified as having a "reduced likelihood of responding to endocrine therapy." Accordingly, alternatives to endocrine therapy, or the use of endocrine therapy in combination with other therapies, are warranted in such an individual to improve the chance of disease-free and overall survival.

The following examples are intended to illustrate but not limit the present invention.

25 EXAMPLE I

This example shows that the tumor suppressor gene RIZ1(PRDM2) is a member of the histone methyltransferase superfamily and a coactivator of female sex hormone receptors. This example also shows that RIZ1 mediates female sex hormone action in vivo, and that loss of its function is involved in the transition to hormone

resistance and hormone independent growth of breast cancer.

Materials and Methods

Plasmids and transient transfections

5 The human RIZ1 and RIZ2 expression vectors and RIZ1 mutant vectors used are described in Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001). The mammalian RIZ1 expression vector p3RIZ1RH4.1 was constructed by cloning the full-length wild type human RIZ1 cDNA into
10 the pcDNA3 vector (Invitrogen). The vector p3RIZ1RH4.1-L965A containing a missense mutation (leucine to alanine) was generated by the Quick-Change mutagenesis kit (Stratagene), and verified by DNA sequencing. Expression vectors of RIZ proteins and expression vectors
15 containing full length mammalian ER α , PR, RAR α , RXR α , VDR, AR, TR and GR were cotransfected into CV-1 cells with an appropriate reporter construct containing a synthetic hormone response element linked to the tk-CAT reporter. The reporter TREpal-tk-CAT containing a
20 synthetic response element for RAR, RXR and TR (Zhang et al., Nature 355:441-446 (1992)) was used to evaluate effects of RAR α , RXR α , and TR, the GRE-tk-CAT (Zhang et al., J. Biol. Chem. 266:8248-8254 (1991)) for GR and AR, the ERE-tk-CAT (Lee et al., Mol. Cell. Biol. 15:4194-4207
25 (1995)) for ER α , the PRE-tk-cat for PR (McKay and Cidlowski, Mol. Endocrinol. 12:45-56 (1998)), and the VDRE-tk-CAT (Agadir et al., Carcinogenesis 20:577-582 (1999)) for VDR.

 A calcium phosphate precipitation procedure was
30 used for transient transfection as described previously (Lee et al., Mol. Cell. Biol. 15:4194-4207 (1995)). Briefly, $0.5-1.0 \times 10^5$ cells/well were seeded in 24-well

plates, and 50-400 ng of RIZ plasmids, 100 ng of expression vectors for NHRs, 100 ng of reporter plasmid, and 100 ng of a β -gal expression vector were mixed with carrier DNA to 1 μ g of total DNA/well. Transfections of
5 MCF-7 cells (2×10^5 cells/well in 6-well plates) used the Effectene Transfection Reagent (Qiagen), according to the manufacture's instructions. Cells were treated with or without the indicated hormone (50 nM for estradiol (E2), and 100 nM for R5020 (synthetic P agonist), all-trans
10 retinoic acid (all-trans-RA), 9-cis retinoic acid (9-cis-RA), 1,25 dihydroxyvitamin D3 (VD3), dihydrotestosterone (DHT), triiodothyronine (T3), and dexamethasone (DEX); all from Sigma except R5020 which was from NEN) for 24 h. and CAT activity was measured as
15 described (Lee et al., Mol. Cell. Biol. 15:4194-4207 (1995)). CAT values were normalized for transfection efficiency by the corresponding β -gal activity.

Plasmids used for GST protein production were constructed by PCR cloning as follows. To express human
20 RIZ1 protein 1-200 residue region, full length human RIZ1 cDNA plasmid was used as template for PCR (Pfu polymerase, Stratagene) by primers hRP109.2 (AAA CCA TGG ATC AGA ACA CTA CTG AG (SEQ ID NO:11)) and RP274 (CCG TAA GCT TCA TGC AGA GGT GAA ATC TGG C (SEQ ID NO:12)). The
25 PCR fragment was cloned into the NcoI and HindIII sites in the vector pKG-PBR (Huang et al., J. Biol. Chem. 273:15933-15940 (1998b)) to generate pKG-HN1. BL-21 cells were transformed by pKG-HN1 to produce the GST-RIZ200 protein. To express RIZ1 protein 1-161
30 residue region, a stop codon was introduced at residue 162 in the plasmid pKG-HN1 by using the Quick-Change mutagenesis kit (Stratagene). The primers used were RP296P1 (CGA GCC AGC GCC CGG AGC TAA GCT TAA GCG GAG CTC CCC (SEQ ID NO:13)) and RP296P2 that has the complement
35 sequence of RP296P1. To generate missense mutant

GST-RIZ200 proteins, mutagenesis by Quick-Change was performed on the pKG-HN1 plasmid template using primers as described previously (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). To generate longer peptide
5 fragments of RIZ1 in bacteria, the N-terminal 520-residue fragment was first cloned into the NcoI and HindIII sites of pKG-PBR vector by PCR to generate the plasmid pKGH205. However, this plasmid did not give high yields of the expected protein product. Next, a stop codon was
10 introduced into this plasmid by Quickchange mutagenesis to generate pKGRIZ332, which then produced high yields of the RIZ1 N-terminus 332-residue peptide. The PCR primers used for mutagenesis are: RP307: GAT TTA TTA GAG GAA tga AAA ACA ACT TCA GAA G (SEQ ID NO:14), and RP308: CTT CTG
15 AAG TTG TTT TTc aTT CCT CTA ATA AAT C (SEQ ID NO:15).

Immunoprecipitation and Immunoblot

MCF-7 and T47-D breast cancer cells were grown in 15 cm dishes in DMEM with 5 % FCS, and 2 mM L-glutamine. Subconfluent cells (4×10^6) were then
20 cultured in DMEM without phenol red, serum or hormones for an additional 3 days, during which media was changed twice daily. Cells were incubated for 24 h with or without hormones (50 nM for E2 and 100 nM for R5020) and infected with an adenovirus vector containing RIZ1
25 (AdRIZ1; at a concentration of 1×10^{10} viral particles/15 cm dish) or the empty vector (AdNull) (He et al., Cancer Res. 58:4238-4244 (1998)). Cells were then grown for an additional 48 h in DMEM without phenol red supplemented with 5 % charcoal-treated FCS (Omega Scientific). Cells
30 were harvested and proceeded to immunoprecipitation and immunoblot essentially as described (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991 (1997)). Antibodies used included RIZ monoclonal antibody 2D7 (Buyse et al., Proc.

Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)), monoclonal antibodies versus ER α (sc-8005, Santa Cruz Biotechnology), progesterone receptor (1A6, DAKO). For histone methylation analysis, anti-dimethyl-H3-K9 and
5 anti-dimethyl-H3-K4 antibodies (Upstate Biotech) were used for immunoblot analysis of acid extracted histones from cells infected with control and AdRIZ1 viruses for 48 hr. For H1 phosphorylation analysis, anti-phosph-H1 and anti-H1 antibody (Upstate Biotech) were used for
10 immunoblot analysis of acid extracted histones.

Mice

RIZ1 $-/-$ mice were generated as described in Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001). Briefly, a PR-domain-encoding cDNA fragment of rat RIZ1
15 cDNA was used to screen a mouse 129Sv genomic library (Stratagene) to obtain mouse genomic DNA. The targeting construct was designed to insert the PGK-neo r -BpA cassette (Soriano et al., Cell 64:693-702 (1991)) into exon 5 and to place the Herpes Simplex Virus TK cassette from
20 PMC1-TK (Mansour et al., Nature 336:348-352 (1988)) external and adjacent to the region of homology. The targeting plasmid was linearized by XhoI digestion and electroporated into D3 ES cells (Gossler et al., Proc. Natl. Acad. Sci. USA 83:9065-9069 (1986)). The ES clones
25 were selected in media containing G418 (150 μ g/ml) and ganciclovir (2.0 μ M). Homologous recombination events were screened by Southern blot analysis with XbaI digestion and hybridization to the 0.5-kb XbaI-BglII 5' probe or with NotI plus EcoRI digestion and hybridization
30 to the 0.5-kb SacII-NotI 3' probe. Five RIZ1 $+/-$ ES cell-lines were microinjected into C57BL/6 blastocysts that were implanted subsequently into the uteri of Balb/c foster mothers. Male chimeric mice generated from two RIZ1 $+/-$ ES clones transmitted the RIZ mutation into

germline. The presence of the RIZ1 mutant allele in the F1 animals was confirmed by Southern blotting and polymerase chain reaction (PCR) analysis of genomic DNA from the tail of animals. RIZ1+/- F1 animals were bred
5 together to generate RIZ1-/- mice.

To examine reproductive functions, female RIZ1-/- mice were bred with male RIZ1+/- mice, female RIZ1+/- mice were bred with male RIZ1-/- mice, and breedings between heterozygous mutants were also
10 performed. The number of litters was recorded, and all mice were routinely measured for body weight throughout the studies.

Analysis of gene expression

For RIZ staining, the RIZ rabbit antiserum
15 KG7.1S (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)) was used at an optimal dilution of 1/200. For ER α staining the polyclonal rabbit anti-ER α antibody (sc-542 Santa Cruz) was used at a dilution of 1/250, and for PR staining the polyclonal rabbit anti-PR
20 antibody (A0098 DAKO) was used at a dilution of 1/100, essentially as described (Tibbetts et al., Biol. Reprod. 59:1143-1152 (1998)). Tissues were fixed in Bouin's solution and processed by routine methods for embeddement in paraffin and sectioning (5 μ m). For RIZ and ER
25 staining, sections were treated with trypsin (Zymed), whereas for PR staining, the sections were subjected to treatment using the Target Retrieval Solution (DAKO), all performed according to the manufacture's instructions. Sections were then incubated with the primary antibody
30 followed with a biotinylated secondary antibody. The localization of the primary antibody was visualized with the imidazole-DAB reaction producing a brown colored stain, followed by hematoxyline counterstaining and

routine processing for bright-field microscopy analysis. Negative controls included omission of the primary antibodies and incubation with the preimmune KG7.1S antiserum, whereas colorectal xenograft tumors infected with AdRIZ1 were used as positive control for RIZ expression (Jiang and Huang, Cancer Res. 61:1796-1798 (2001)).

For RT-PCR analysis of RIZ gene expression, tissues from 7-week old RIZ1 +/- mice were pulverized in liquid nitrogen and total RNA was isolated using the TRIZOL reagent (Gibco BRL). cDNA was synthesized using the first strand cDNA synthesis kit (Gibco BRL). Oligos RP260 (5'-CTC ATT CAT CTA AGA AAG GTG G-3'; SEQ ID NO:16) + RP259 (5'-TGA TTC CAG GTC ACT TCA GG-3'; SEQ ID NO:17) and RP170 (5'-GAA GCC AAA GGC CTC TCA TC-3'; SEQ ID NO:18) + K05 (5'-AGA CTC TGG CTG AGG TAC C-3'; SEQ ID NO:19) were used in standard PCR conditions encompassing 30 cycles at an annealing temperature of 59°C to amplify the RIZ1+2 and RIZ1 specific fragments, respectively.

Analysis of target organs in response to hormone treatments

To investigate E2-mediated increments in proliferation and hyperemia of uteri and changes in the cellular organization of the vaginal epithelium (Xu et al., Science 279:1922-1925 (1998)), 8-week old female RIZ -/- and RIZ +/- mice were ovariectomized (OVXed). At day 15-17 following OVX, mice were treated with S.C. injections of E2 (0.8 ng/g/day; Sigma) or with the vehicle (0.1 ml corn oil) alone for three days. The mice were then sacrificed at day 18 and the uterine wet weight was measured, and vaginal tissue was collected. The tissues were processed as above and vaginal specimens were stained with routine H & E and uterine specimens for

PR expression. The thickness of the vaginal epithelium and the cornified layer was photographed and measured as described in Figure 4 using the Spot 3.2.4. software (Diagnostic Instruments).

5 To evaluate the P- and E2-mediated decidual response a previously described protocol was used (Lydon et al., Genes Dev. 9:2266-2278 (1995)); Xu et al., Science 279:1922-1925 (1998)). Briefly, 8 week old female mice were OVXed and at day 10-12 following OVX,
10 mice were treated with S.C. injections of E2 in corn oil (100 ng/day), then treated with P (1 mg/day; Sigma) + E2 (6.7 ng/day) from day 16-23. Six h after the third P + E2 injection on day 18, the left uterine horn (right horn served as a control) was traumatically stimulated by
15 insertion of a burred needle proximal to the cervix and longitudinally scratching the entire length of the uterine horn antimesometrially. The mice were sacrificed on day 23, six hour after the last P + E2 injection, and the wet weights of the left and right uterine horns were
20 measured.

 To measure effects on mammapoiesis by normal development, pregnancy, and female sex hormone treatment the following protocols were used (Xu et al., Proc. Natl. Acad. Sci. USA 97:6379-6384 (2000); Xu et al., Science
25 279:1922-1925 (1998)). To determine the effects of normal pubertal development on mammary gland growth, 7 week old virgin female mice were sacrificed. Additionally, pregnant female mice at day 19 of first pregnancy were sacrificed. To examine the effects of E2
30 + P treatments on mammary gland development, 8 week old female mice were OVXed and at 14 days following OVX treated with S.C. 21-day releasing hormone pellets containing 0.1 mg of E2 and 10 mg P4 or S.C. placebo pellets (Innovative Research of America). On day 35, the

mice were sacrificed and the uterus was wet weighed and analyzed microscopically to ensure actual hormone release. In all cases, whole mounts were prepared from the fourth mammary gland and carmine-stained according to standard procedures (Evans et al., Oncogene 19:989-991 (2000)), and the relationship between mammary ducts and mammary fat pad, extent of branching as well as number of branches were investigated microscopically.

In order to evaluate the response to testosterone, twelve week old male mice were orchiectomized (ORCed) and testicles were wet weighed (Xu et al., Science 279:1922-1925 (1998)). Nine days following ORC, mice were treated during days 9-15 with S.C. injections of testosterone (3 mg/kg/day, Sigma) or vehicle alone (0.1 ml corn oil). On day 16, mice were sacrificed and the prostate and the pars prostate of the urethra (for technical reasons) were removed and wet weighed.

Statistical analysis

Student's unpaired t-test was used for statistical evaluation of means, with $P < 0.05$ considered to be significant. All results are expressed as mean \pm SEM (standard error of the mean) or mean \pm SD (standard deviation, when stated). Fisher's exact test was used for evaluation of the difference between ER-negative and ER-positive breast cancers, with $P < 0.05$ considered to be significant.

Methylation assays

Methylation reactions (30-40 μ l) contained 20 mM Tris-HCl (pH8.0), 0.2 M NaCl, 0.4 mM EDTA, affinity-purified GST-RIZ1 or immunoprecipitation

products, free histones (20-40 µg) purified from acid extraction of human cancer cells (A549 or U2OS), and 3 µl (1.65 µCi and 21 pmole) [methyl-³H]-adenosyl-methionine (Amersham-Pharmacia). Nuclear extracts were prepared according to standard procedures and used for immunoprecipitation. Antisera used for immunoprecipitation were anti-KG7.1S and preimmune serum as described previously (Buyse et al., Proc. Natl. Acad. Sci. U.S.A. 92:4467-4471 (1995)). Similar HMT activity has also been observed with immunoprecipitates using a different serum (data not shown), 1715, which was described previously (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). Various amount of SAH (Sigma) were added in some methylation reactions.

To determine the methylated residues on H3 and H1, H3 N terminus 1-20 residue peptides (Upstate Biotech), non-acetylated and acetylated on K9 and K14, and H1 N terminus 15-37 and 12-31 peptides were used as substrates for in vitro methylation reactions as described above. The reactions were resolved on Tris-Tricine 10-20% gel (Invitrogen) followed by fluorography. The ³H-labelled H3 or H1 peptides were purified by HPLC and sequenced from the amino termini in an Applied Biosystems model 477A Protein Sequencer. After conversion, the samples were collected for determination of radioactivity by scintillation counting.

Histones from nocodazole treated cells were isolated by acid extraction of U2OS cells treated for 48 hour with 50 ng/ml nocodazole. Histones (20 mg) were treated with lambda protein phosphatase (400 U) (New England Biolabs) for 1 hr at 30 °C in buffer. Control histones were treated the same except that phosphatase was omitted from the incubation mixture.

Results

RIZ1 is a specific coactivator of ER and progesterone receptor

RIZ1 has been shown to be a coactivator of
5 estrogen receptor (ER) (Abbondanza et al., Proc. Natl. Acad. Sci. USA 97:3130-3135 (2000); Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). To better understand the coactivator function of RIZ1, the effects of RIZ1 on ER were compared with its effects on several
10 other NHRs. As shown in Fig. 1A, RIZ1 enhanced ER and progesterone receptor (PR) function in a ligand- and dose-dependent manner. In contrast, RIZ1 had no major effects on the activities of androgen receptor, retinoic acid receptor α (RAR α), retinoic X receptor α (RXR α),
15 thyroid hormone receptor (not shown), glucocorticoid receptor (not shown), and vitamin D receptor (not shown). Similar to what has been found with ER (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)), RIZ2 and RIZ1 missense mutants (C106Y and I188V) failed to coactivate
20 progesterone receptor (Fig. 1A). The introduction of a point mutation in the LXXLL motif of RIZ also produced a protein unable to act as a coactivator for progesterone receptor (Fig. 1A). Consistent with RIZ1 effects on ER and progesterone receptor, protein complexes containing
25 RIZ1 and these receptors were detected by a coimmunoprecipitation assay (Fig. 1B). Thus, RIZ1 is a specific coactivator of estrogen receptor and progesterone receptor but not for other nuclear hormone receptors, and the intact PR/SET-domain of RIZ1 is
30 essential for its function.

To confirm the expected RIZ expression in the target organs of E2 and progesterone, RIZ expression was determined by immunohistochemistry. The gene products

displayed nuclear staining in the uterus, including both glandular and luminal epithelium as well as stromal and myometrial cells, similar to the expression of ER (Fig.1C). Additionally, RIZ is expressed in both mammary and vaginal epithelium. For these experiments, the serum anti-KG7.1S, which recognizes both RIZ1 and RIZ2, was used (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991 (1997)). Positive results from this antibody likely reflect expression of both proteins because they are coexpressed in most tissues (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995); Liu et al., J. Biol. Chem. 272:2984-2991 (1997)). Nonetheless, by RT-PCR analysis using primer sets that can differentiate RIZ1 from RIZ2, it was confirmed that RIZ1 was indeed expressed in various target tissues of female sex steroid hormones (Fig. 1D).

Partial hormone resistance in target organs of RIZ1 deficient female mice

The RIZ1 mutant mice are deficient in RIZ1, but not RIZ2, and therefore lack specifically any PR-domain related functions of the gene (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). These animals are viable and fertile and show no gross developmental defects (Steele-Perkins et al., supra (2001)). To determine the physiological role of RIZ1 in steroid hormone action, uterine growth in response to E2 was first examined in ovariectomized (OVXed) mice (Lubahn et al., Proc. Natl. Acad. Sci. USA 90:11162-11166 (1993)). Wild-type or heterozygous RIZ1 mutant mice responded to E2 treatment with a 5.3 ± 0.5 and 5.4 ± 0.4 fold increase in uterine wet weight. Uteri of homozygous RIZ1 mutants showed a smaller increase of about 3.7 ± 0.4 fold ($P < 0.001$ for +/+ vs. -/-; Fig. 2A and B). Uterine

response to mechanical traumatization (decidual stimulation) is mainly a progesterone receptor-dependent process (Lydon et al., Genes Dev. 9:2266-2278 (1995)). OVXed mice were treated with a high dose of progesterone and a low dose of E2, followed by mechanical stimulation of the left uterine horn of each animal. The unstimulated left uterine horn served as a control. The decidual response resulted in an increase in the uterine horn size and was consistently observed in the stimulated left uterine horn in wild-type mice. Only a partial response was observed in the uterine horn of the RIZ1 null mutant (Fig. 2C). These results suggest that RIZ1 is required for maximal uterine response to E2 and progesterone in vivo.

Vaginal cornification in response to a three-day treatment of E2 was examined in OVXed mice. The hormone produces an increased vaginal thickening and cornification of the epithelium (Lubahn et al., supra (1993)), which was consistently seen in the wild-type mice (Fig. 2D,E). RIZ1 null female mice, however, failed to show a maximal such response, thus suggesting that RIZ1 is required for vaginal response to E2 in vivo.

The epithelial and stromal compartments of the uterus were then analyzed for E2-induced changes in progesterone receptor expression. Such a regulation via ER is highly compartment-specific and mimics the changes in the uterus during the estrous cycle (Tibbetts et al., Biol. Reprod. 59:1143-1152 (1998)). Progesterone receptor staining in OVXed RIZ1 +/+ and -/- mice treated with vehicle was apparently similar and showed strong immunoreactivity in almost all cells of both the luminal (LE) and glandular epithelium (GE), whereas only a fraction of stromal and myometrial cells were positive (Fig. 2G, data not shown). As expected, E2 treatment of

wild type animals had dual effect on progesterone receptor expression, reducing the levels in the LE and increasing the levels in the stromal and myometrial compartments. Immunoreactivity in the GE was unchanged, consistent with previous findings showing that E2 alone does not regulate progesterone receptor expression in this compartment (Fig. 2F,G) (Tibbetts et al., supra (1998)). Upon E2 treatment of RIZ1 deficient animals, the decrease of progesterone receptor levels in the LE was nearly 8-fold less than in wild type tissues, and the increase of the receptor level in the stroma and myometrium was nearly 2-fold less than normal. These findings suggest that RIZ1 plays a role in the E2 regulation of progesterone receptor expression in the uterus.

Steroid hormones play an important role in mammapoiesis (Hennighausen and Robinson, Genes Dev. 12:449-455 (1998)). Both E2 and progesterone are essential for alveolar development during pregnancy. In 7-week-old wild-type females, mammary ducts had started to grow into the mammary fat pad but with no apparent differences in the age-matched RIZ1 null mutants (Fig. 3A and B). However, when stimulated by pregnancy, alveolar structures in wild-type mammary glands were highly developed and appeared on all ductal sections, filling the interductal spaces. In the RIZ1 mutant mammary glands, alveoli were much less developed in terms of number and size of alveoli, and fewer alveoli were observed at the ends of ducts at the same stage of pregnancy (Fig. 3C through F). Although mammary glands of RIZ1 null mutants can still produce milk, these results suggest that RIZ1 is required for normal mammary ductal elongation and alveolar development in vivo during pregnancy. Mammary gland development in response to E2 and progesterone treatment in OVXed adult mice was also

examined. E2 and progesterone stimulated a complex ductal arborization and extensive alveolar formation in mammary glands of wild-type mice, which mimics a stage of mammapoiesis in early pregnancy. In the mammary glands
5 of RIZ1 mutant mice, only partial ductal growth was observed after hormone treatment (Fig. 3G through J). Thus, RIZ1 seems to be required for efficient proliferation and differentiation of the mammary gland in response to E2 and progesterone.

10 *Normal steroid hormone response in RIZ1 deficient male mice*

Although RIZ1 is expressed in target tissues of male steroid hormones such as testis (Buyse et al., Proc. Natl. Acad. Sci. USA 92:4467-4471 (1995)), it does not
15 appear to serve as coactivator for androgen receptor (Fig. 1A). To study this further, prostate growth was measured in orchiectomized male mice after they were treated with androgen. Similar to female mice, no difference in total body weight was noted in RIZ1 null
20 mutant male animals (Table 1).

Table 1

		Mean \pm S.D. (number of mice weighed)		
		5 weeks	8 weeks female	12 weeks female
RIZ1-/-	Female -			
	16.83 \pm 1.64 (11) 83	21.28 \pm 2.70 (32)	24.27 \pm 2.21 (13)	
	Male -			
	19.24 \pm 2.25 (7)			
RIZ1+/-	Female -			
	16.83 \pm 2.01 (15)	20.49 \pm 2.70 (12)	24.51 \pm 3.22 (23)	
	Male -			
	20.00 \pm 2.56 (15)			
5 RIZ1+/+	Female -			
	18.52 \pm 1.55 (5)	20.37 \pm 2.13 (31)	21.71 \pm 3.49 (13)	
	Male -			
	20.61 \pm 2.72 (7)			

Eight days after orchiectomization, prostates in both wild-type and mutant animals regressed. Injection of testosterone for 7 days stimulated prostate growth in wild-type animals as well as in RIZ1 null animals. Wild-type and RIZ1 null mutant animals had similar ratios of the weight of prostate and urethra to body weight (1.19 ± 0.03 vs. 1.14 ± 0.03 ; $n=10$ and 9 mice for $+/+$ and $-/-$, respectively; $p=0.23$). They also had similar ratios of testis weight to body weight (7.7 ± 0.4 vs. 7.4 ± 0.4 ; $n=14$ for both genotypes; $p=0.58$). Thus, tissue responses to testosterone are not significantly affected in mice lacking RIZ1, consistent with the lack of effect of the gene on androgen receptor transactivation functions (Fig. 1A).

20 *Reduced litter size by RIZ1 deficient female mice*

The reproductive abilities of RIZ1 mutant mice were assessed. Female RIZ1-/- mice were bred with male RIZ1+/- mice and as controls, female RIZ1+/- mice were bred with male RIZ1-/- mice. Also, breedings between

heterozygous mutants were conducted. The average litter size from breeding RIZ^{-/-} female x RIZ1^{+/-} male was 6.6 ± 3.2 (S.D.; n = 35 litters) but the average litter size was 8.4 ± 2.0 (n = 20 litters) for litters of breeding RIZ1^{+/-} female x RIZ1^{-/-} male (P < 0.01), and 8.0 ± 2.2 (n = 50 litters) for litters of breeding between heterozygous mice (P < 0.02). The results indicated a slightly compromised reproductive ability associated with the female, but not male, mice deficient in RIZ1, consistent with the above results showing defective tissue responses to steroids in female but not male animals.

Regulation of ER-dependent transcription in breast cancer by RIZ1 and RIZ2

The above results suggest a physiological role of RIZ1 in E2 response of normal tissues. In view of RIZ1's tumor suppressor role, the question was posed whether RIZ also plays a role in the E2-response of breast cancer cells. RIZ1 is often lost but RIZ2 is uniformly present in breast cancer (He et al., Cancer Res. 58:4238-4244 (1998); Jiang and Huang, Histol. Histopathol. 15:109-117 (2000)). RIZ2 does not serve as a coactivator of ER and can actually neutralize the coactivator function of RIZ1 (Steele-Perkins et al., Genes Dev. 15:2250-2262 (2001)). Therefore, the effects of overexpressing RIZ1 and RIZ2 in MCF-7 were examined in breast cancer cells that express ER and RIZ1 (He et al., supra (1998)). When cotransfected with the ERE-tk-CAT reporter into MCF-7 cells, RIZ1 enhanced estrogen-induced reporter activity (Fig. 4A). In contrast, E2 induced reporter gene transcription was suppressed by RIZ2 expression. In CV-1 cells that lack ER, reporter gene expression was not affected by RIZ1, RIZ2, and E2 (Fig. 4B). The data suggest that RIZ1 can sensitize breast

cancer to E2 and that decreasing RIZ1 activity, as achieved by RIZ2 expression, can inhibit E2 sensitivity of breast cancer.

RIZ1 gene silencing in hormone resistant breast cancer

5 If RIZ1 appears to facilitate E2-mediated normal and breast cancer growth as indicated by the above studies, why is it also a tumor suppressor and often silenced in breast cancer (He et al., supra (1998))?
Upon re-analysis of the published data, it was found that
10 RIZ1 loss is related to hormone resistance (as represented by ER status) among those breast cancer cell lines examined (Table 2).

 A methylation-specific PCR assay was developed to examine RIZ1 promoter methylation, which showed that
15 DNA methylation is a good indicator of lost or decreased RIZ1 mRNA expression in tumor cell lines and tissues (Du et al., Cancer Res. 22:8094-8099 (2001)). Methylation of RIZ1 promoter was found to be common in breast cancer tissues, occurring in 11 of 25 breast cancer tissues
20 examined. Except for BT20 and ZR75-1 cell lines, the mRNA expression data in Table 2 are from (He et al., supra (1998)). Expression levels below 10% of normal were scored negative (-).

Table 2

	RIZ1		ER
	Methylation	mRNA	
5	MB468	+	-
	MCF-7	-	+
	MB-435	+	-
	T47-D	-	+
	MB231	+	-
10	BT474	-	+
	SKBR3	+	-
	BT549	-	-
	BT20	-	+

To address the relationship between RIZ1 and hormone responsiveness of breast cancer tissues, the ER expression status was examined for 18 of the 25 samples for which tissue sections were available. ER expression was determined by immunohistochemistry (below 10% positivity was considered negative) and was scored in a double-blind fashion without knowledge of the methylation status of these samples. The results showed the same trend as previously found in cell lines that ER negative tumors tend to be RIZ1 negative (Table 3).

Table 3

Expression	ER (-)		ER (+)	
	RIZ1 (-)	RIZ1 (+)	RIZ1 (-)	RIZ1 (+)
# tumor cases	83% (5/6)	17% (1/6)	42% (5/12)	58% (7/12)
# cell lines	67% (4/6)	20% (2/6)	0%	100% (4/4)
# Total	75% (9/12)	25% (3/12)	31% (5/16)	
	69% (11/16)			

Thus, 5 of 6 ER-negative cases were RIZ1 negative compared to 5 of 12 ER-positive cases. Together with data from cell lines, these results shows that there is a significant association between RIZ1 silencing and ER-negative breast cancers (RIZ1 silencing found in 9 of 12 ER-negative samples versus 5 of 16 ER-positive samples, $P < 0.05$). The results also reveal that nearly half of ER-positive cancer tissues were RIZ1 negative (5 of 12), indicating a potential role of RIZ1 silencing in the development of hormone resistance of ER-positive breast cancers. Together, these studies indicate a dual status of RIZ1 in breast cancer that is linked with the presence or absence of ER, and in turn, the early or late stage of the disease. The dual status of the gene appears consistent with its dual role as a tumor suppressor and an ER/E2-dependent tumor promoter (see discussion below).

Histone methyltransferase activity of RIZ1 and regulation by estrogen

The results of animal knock out studies presented here and previously (Steele-Perkins et al.,
5 supra (2001)), as well as the dual role of RIZ1 in breast cancer discussed above, suggest a physiological role of RIZ1 as an ER-coactivator/growth promoter and a tumor suppressor. Because it is only the PR domain of RIZ1 that is missing in these animals or in tumors, owing to
10 normal expression of RIZ2, it was asked whether RIZ1 has an activity that may explain its dual function as an ER-coactivator/growth promoter and tumor suppressor.

Recombinant GST fusion proteins of RIZ1's N-terminus PR domain region were prepared in bacteria.
15 The recombinant proteins were purified and assayed for histone methyltransferase activity using histones as substrates and S-adenosyl-[methyl-³H]-L-methionine as methyl group donor. Reaction products were separated by SDS-PAGE and visualized by flurography. Specific
20 labeling of histone H4 was observed in the presence of purified GST product consisting of residues 1-200 of RIZ1 (Fig. 5A). GST alone or a protein consisting of residues 1-161 lacked HMT activity. Furthermore, missense mutations found in tumors, C106Y, I188V, and A159V,
25 abolished HMT activity. The results suggest that RIZ1 has HMT activity, which requires the conserved PR-domain (residues 30-160) as well as the non-conserved residues C-terminal to the PR-domain (residues 162-200).

It was next asked whether the HMT activity of
30 the PR domain may be modulated by additional sequences of RIZ1 by testing longer RIZ1 N-terminal fragments made in bacteria. A GST fusion protein expressing the N-terminal 332-residue peptide showed similar activities to the

N-terminal 200-residue peptide in methylating H4 (Fig. 5B). An attempt to test an even longer peptide (520 residue) in bacteria was not successful because of low yield of protein. The result suggests that residues
5 200-332 may not intrinsically contribute to HMT activity but could not exclude other regions of RIZ1 (333-1719) from playing role in modulating the HMT activity of the N-terminal 200-residue region.

A domain located at the C-terminus of RIZ1
10 protein (amino acids 1514-1680) was previously defined that can bind to the PR domain of RIZ1 (Huang et al., J. Biol. Chem. 273:15933-15940 (1998)). It was next asked whether this domain, termed PBD or PR-binding domain, can regulate the PR HMT activity. The previously described
15 GST fusion protein, GST-hRIZ1(1514-1680) or now termed GSTRIZPBD, was examined for its effects on the HMT activity of GSTRIZ332. Unexpectedly, GSTRIZPBD protein alone showed HMT activity, specifically on H4, suggesting that the RIZ PBD domain represents a novel catalytic
20 motif of methyltransferase. A synergistic enhancement (more than additive effects) of HMT activity was observed when GSTRIZ322 and GSTRIZPBD were both present in HMT reactions, as indicated by the increased methylation of H4 and the methylation of a new substrate with a
25 molecular weight similar to that of H1 (Fig. 5C). This result suggests that the PR domain HMT activity can be modulated by other regions of RIZ1, such as the PBD domain.

Enzyme activity of full-length RIZ1 protein
30 from mammalian cells was next assayed. Human osteosarcoma U2OS cells, which express little RIZ1 (He et al., Cancer Res. 58:4238-4244 (1998)), were infected with AdRIZ1 virus or control AdNull virus and nuclear extracts from virus infected cells were immunoprecipitated with

RIZ serum anti-KG7.1S or preimmune serum.

Immunoprecipitated products by RIZ1 serum from RIZ1-expressing extracts caused methylation of H1, H3, and H4 (Fig. 6A). A similar result was also obtained using a different RIZ1 antiserum, 1715 (data not shown). As controls, HMT activity was not detected for immunoprecipitates of preimmune serum or when RIZ1 negative nuclear extracts were used for immunoprecipitation.

10 The data indicate that RIZ1 protein of mammalian cells has HMT activity, which is consistent with the activity of truncated proteins obtained from bacteria (Fig. 5). However, full length protein appears to target more histone substrates than truncated proteins from bacteria, probably because it has sequences or
15 modifications or both that are missing in bacteria expressed proteins. The results are consistent with other regions of RIZ protein regulating PR domain HMT activity.

20 In view of RIZ1's role in tumor suppression, it was determined whether S-adenosylhomocysteine (SAH), an analog inhibitor of SAM-dependent methyltransferases whose abnormal accumulation is linked with carcinogenesis, can inhibit RIZ1 enzyme activities. The
25 effect of SAH on the in vitro methylation of histones by immunoprecipitated RIZ1 was tested. A methylation assay was performed in the presence of 0, 0.5 and 5 μ M of SAH and 0.42 μ M of 3H-SAM. Histone methylation by RIZ1 was partially inhibited by 0.5 μ M of SAH and completely
30 inhibited by 5 μ M of SAH (Fig. 6B). The results show that SAH is an inhibitor of RIZ1 methyltransferase.

 The methylated lysine residues at position 4 and 9 of histone H3 are the best-characterized histone

lysine methylations with regard to their biological function. Methylation of H3-K9 is associated with gene repression and heterochromatin formation, whereas methylation of H3-K4 is often linked to gene activation
5 (Noma et al., Science 293:1150-1155 (2001); Rea et al., Nature 406:593-599 (2000); Strahl et al., Curr. Biol. 11:1-5 (2001)). Each of the two lysine residues can be targeted by several different SET domain HMTs, which are either K4 or K9 specific but not both, and often have
10 non-H3 substrates such as H1 (Nishioka et al., Genes & Dev. 16:479-489 (2002); Rea et al., Nature 406:593-599 (2000); Tachibana et al., J. Biol. Chem. 276:25309-25317 (2001); Wang et al., Mol. Cell. 8:1207-1217 (2001a)). To determine whether RIZ1 may belong to such a group of
15 H3-K4 or H3-K9 specific HMTs may immediately explain how RIZ1 functions in transcription. Therefore, it was determined whether RIZ1 may have any activity toward H3-K4 or H3-K9. First, it was determined whether RIZ1 can alter the methylation of these residues in vivo in
20 mammalian cells. Histones were extracted from U2OS cells infected with AdRIZ1 or control virus and analyzed by western blot analysis using antibodies that recognize specifically H3-K9 methylation or H3-K4 methylation. The result shows that RIZ1 increased H3-K9 methylation but
25 did not affect H3-K4 methylation (Fig. 6C), consistent with H3-K9 but not H3-K4 as a potential target of RIZ1 methyltransferase.

To show that RIZ1 can directly methylate H3-K9, peptide methylation and sequencing experiments were
30 performed. A peptide consisting the N-terminal 1-20 residues of H3 was indeed methylated in vitro by immunoprecipitated RIZ1 protein using S-adenosyl-[methyl-3H]-L-methionine as methyl donor (Fig. 6D). In contrast, a peptide containing acetylated K9 and
35 K14 was not methylated, consistent with K9 but not K4 as

a target of RIZ1. The migration of the peptide as a doublet has also been observed by others, and may be due to the poor resolution of SDS-PAGE. The methylated peptide was next purified by HPLC and subjected to
5 peptide sequencing analysis. Purified peptides were sequenced from the amino termini and the radioactivity of each of the sequenced residues was determined by scintillation counting. A single radioactive peak at lysine 9 was observed (Fig. 6E). The results suggest
10 that H3-K9, but not H3-K4, is a target of RIZ1 methyltransferase.

Next, the possible methylation site on H1 was determined. A H1 peptide (aa 15-37) was methylated by RIZ1 in vitro, which was then subjected to HPLC
15 purification and sequencing analysis. Repeated methylation and sequencing analysis showed that lysine 25 was methylated by RIZ1 (Fig. 6F).

The capacity to methylate H3 in a K9 specific manner suggests a role for RIZ1 in transcriptional
20 repression, which appears to be in conflict with RIZ1's role as an ER coactivator. To help understand this apparent paradox, the estrogen effects on RIZ1 HMT activity were examined. Nuclear extracts from control and E2 treated MCF-7 cells were immunoprecipitated by
25 RIZ1 serum anti-KG7.1S or preimmune serum. As expected, HMT activity (labeling of H3 and H1) was detected in the immunoprecipitates of RIZ1 serum but not preimmune serum (Fig. 7A, lanes 1 and 3). Interestingly, estrogen treatment decreased H3 methylation but maintained or
30 slightly enhanced H1 methylation by RIZ1 (Fig. 7A, lane 4). RNase protection analysis did not reveal altered RIZ1 or RIZ2 gene expression by E2 treatment of MCF-7 cells (not shown). Furthermore, E2 also similarly regulated the HMT activity of overexpressed RIZ1 protein

in AdRIZ1 virus infected MCF-7 cells (Fig. 7A, lanes 5-6); cells infected with control virus showed similar results as non-infected cells (not shown). Thus, E2 appears to regulate RIZ1 HMT activity in a selective way, inhibiting specifically methylation of H3 but not H1. The data suggest that E2 may turn RIZ1, possibly with a dual role of transcriptional repressor and activator, into a transcriptional activator by selectively turn off its repressor activity as represented by H3 methylation while enhancing its putative activator activity, presumably represented by H1 methylation.

Although the function of H1 methylation remains to be characterized, the results here suggest, at least, that RIZ1 methylation of H1 is unlikely to be similar to H3-K9 methylation, in view of the contrasting behavior of these two methylation activities of RIZ1. Because H1 phosphorylation has been linked with transcriptional activation (Bhattacharjee et al., Mol. Cell. Biol. 21:5417-5425 (2001); Dou and Gorovsky, Mol. Cell. 6:225-231 (2000)), it was next asked whether E2 or RIZ1 can enhance H1 phosphorylation. Histones were extracted from MCF7 cells infected with AdRIZ1 or control virus and analyzed by western blot analysis using antibodies that recognize specifically phosphorylated H1. The result shows that both E2 and RIZ1 increased H1 phosphorylation (Fig. 7B), suggesting that E2 or RIZ1 may function to stimulate transcription in part by mediating enhanced H1 phosphorylation. That RIZ1 methylation of H1 was positively correlated with H1-phosphorylation suggests that such methylation is correlated with transcriptional activation.

Next, the methyl acceptor potential of phosphorylated H1 with non- or hypophosphorylated H1 was compared. Histones were isolated from cells arrested at

G2/M by nocodazole, which were enriched for phosphorylated H1, and used as substrates for in vitro methylation by RIZ1. H1 from nocodazole treated cells appeared to serve as a poor substrates compared to H1
5 from control cells (not shown). This result indicates either that H1 isolated from nocodazole treated cells was both phosphorylated and methylated or that phosphorylation of H1 may prevent its methylation. To resolve this, histones were treated with phosphatase
10 prior to use as methylation substrates. Phosphatase treatment removed a significant amount of phosphate on H1 but did not affect its ability to serve as methylation substrates (not shown). Together, these results suggest that phosphorylated H1 is associated with a higher extent
15 of methylation than non- or hypophosphorylated H1 and that methylated H1 is associated with a higher degree of phosphorylation than non- or hypomethylated H1.

The time course response of RIZ1 methylation activity to E2 treatment was then studied. A decrease of
20 RIZ1 H3 methylation activity was found at 4 hr post E2 treatment, which stayed at low levels thereafter (not shown). Because E2-induced cell proliferation is not known to occur prior to at least 8 hr of treatment, the data suggest that inhibition of RIZ1 H3-methylation
25 activity by E2 is unlikely a result of cell proliferation.

The observation that E2, a growth factor, has a sustained inhibitory effect on RIZ1 methylation of H3 but not H1 suggests that H3 and H1 methylation activity of
30 RIZ1 may be oppositely linked with cell growth. To further study the contrasting behavior of H1 and H3 methylation, regulation of RIZ1 HMT activity by cell growth was examined, using
12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment of

U937 myeloid leukemic cells as a model system. U937 cells undergo growth arrest and terminal differentiation into monocytes when exposed to TPA. This cell model was selected for study because it has been shown in these
5 cells that RIZ gene expression is only marginally regulated by TPA. A slight increase (less than 2 fold) in RIZ1 mRNA, but no change in RIZ2 levels, was not observed until after 3 or more days of TPA treatment (Gazzerro et al., Mol. Med. 7:552-560 (2001)). Thus, any
10 significant changes in HMT activity, especially a decrease, may not be attributed to changes in RIZ1 protein amounts, at least within the first three days of treatment. The TPA effects on U937 cell growth and differentiation were confirmed by counting cell numbers
15 and examining cell morphology (not shown). It was also confirmed that RIZ gene expression was not significantly altered within the first 3 days of TPA treatment. By assaying the HMT activity of RIZ1 immunoprecipitates from nuclear extracts, it was found that H3 methylation was
20 increased, whereas H1 methylation was slightly decreased at 24 hr following TPA treatment (not shown). Similar results were observed at 3 days or 7 day spost TPA treatment, although H3 methylation activity was noted to be lower than that at 1 day post treatment. No
25 significant change in HMT activity was noted for any treatment periods of less than 24 hr. The change at 24 hr post TPA treatment was well before any significant growth arrest had taken place, suggesting that such a change is unlikely a result of growth arrest. The
30 results suggest that the H3 methylation activity of RIZ1 is linked with growth arrest, whereas its H1 methylation activity is associated with cell proliferation, confirming the above described contrasting behavior of these activities in response to E2.

Discussion

The above results show that female mice deficient in RIZ1 display an impaired response to E2 and progesterone, and reduced reproductive abilities. RIZ1 shows coactivator activity for ER and progesterone receptor but no or weak activity for other NHRs examined. Furthermore, the results suggest that a coactivator (RIZ1) may serve as both an E2-dependent growth promoter in early stage breast cancer and a tumor suppressor in preventing breast cancer progression to an estrogen independent state. Estrogen mediated transactivation in breast cancer cells is suppressed by a RIZ1 antagonist (RIZ2), and hormone resistant breast cancers more commonly carry a silenced RIZ1 gene than hormone sensitive breast cancers. These data suggest that RIZ1 has HMT activity, which is mediated by two independent but interacting catalytic motifs. The HMT activity of the PR domain is linked to both its ER-coactivator function and tumor suppressor function. Cancer-associated mutations in the PR-domain, which inactivate its coactivator function, impair the HMT activity. Also, the HMT activity of RIZ1 can be regulated by E2, in a fashion consistent with RIZ1's role as an ER-coactivator.

The specificity of RIZ1 in its interaction with female sex NHRs contrasts to members of the SRC-1/p160 family members, which seem more promiscuous in this respect. Thus, one may expect more subtle phenotypes for RIZ1 deficient mice versus SRC-deficient animals. Indeed, this is the case even when comparing to mice deficient in SRC-1, which shows the mildest phenotypes among animals deficient in a SRC member (Wang et al., Proc. Natl. Acad. Sci. USA 97:13549-13554 (2000); Xu et al., Proc. Natl. Acad. Sci. USA 97:6379-6384 (2000); Xu

et al., Science 279:1922-1925 (1998)). While SRC-1 and RIZ1 deficient female animals show similar reduced sensitivity to female sex hormones, SRC-1 mutation, unlike RIZ1, also affects mammary duct growth during puberty in virgin mice and hormone response in males. The partial hormone resistance phenotypes observed in RIZ1 or SRC-1 deficient mice likely reflect overlapping functions of RIZ1 or SRC-1 related proteins, for which there are many. Or, it may be related to an additive effect of RIZ1 and SRC-1 on ER function. Reduced H3-K9 methylation, as induced by E2 inhibition of RIZ1, would, in and of itself, facilitate transcription activation by reducing the binding of the heterochromatin protein HP1 to methylated K9 on H3 (Bannister et al., Nature 410:120-124 (2001); Lachner et al., Nature 410:116-120 (2001)). In addition, transcription would be further enhanced by the acetylation of the methyl-free H3-K9 by SRC-1 or its related HATs.

The partial reduction in tissue response to steroids in RIZ1 deficient mice was accompanied by a corresponding reduction in target gene expression. E2 induction of progesterone receptor gene expression in the stroma and myometrium compartments of the uterus was reduced in RIZ1 deficient mice (Fig 2F-G). The result suggests a physiological role of RIZ1 in facilitating ER-transcriptional activation function and that such a coactivator function is likely responsible for the observed tissue response to E2.

While the overall tissue response to steroids is only partially reduced by RIZ1 deficiency, this does not necessarily indicate that RIZ1 is unable to serve a unique and non-redundant function in facilitating NHR function. It is possible that at the molecular level, RIZ1 may be uniquely involved in controlling expression

of certain target genes. Indeed, estrogen repressed progesterone receptor gene expression nearly 20-fold in the luminal epithelial cells of the uterus in the presence of RIZ1 but only 2-fold in its absence (Fig. 2F-G). Although ER/E2 appear to largely use RIZ1 as a coactivator, this result further suggests that it can also employ it as a corepressor in certain circumstances. It is possible that ER/E2 may enhance RIZ1 methylation of H3-K9 in certain cellular contexts.

That RIZ1 appears required for E2 induced tissue growth contrasts with its tumor suppressor role. To help understand the apparent paradox, the role of RIZ1 in breast cancer was studied. RIZ1 overexpression enhanced ER-dependent transactivation of reporter gene whereas overexpressing a RIZ1 antagonist, RIZ2, inhibited the transactivation function of endogenous ER in breast cancer cells. Thus, RIZ1 inactivation maybe associated with decreased hormone response in breast cancers. Consistently, RIZ1 silencing was shown to be significantly more common in E2 resistant or ER-negative comparing to ER-positive breast cancers. It is logical to expect that alteration of ER coactivators may play a role in the hormone response phenotypes of breast cancers. This idea has been pursued by several previous studies, which found overexpression of SRC3 (AIB1) and SRA in mostly ER-positive breast cancers (Anzick et al., Science 277:965-968 (1997); Kurebayashi et al., Clin. Cancer Res. 6:512-518 (2000); Murphy et al., Cancer Res. 60:6266-6271 (2000); Thenot et al., Mol. Cell. Endocrinol. 156:85-93 (1999)). As might be anticipated, there is a quantitative relationship between ER concentration in breast cancer tissue and response to E2 (Allegra, Semin. Oncol. 10:23-28 (1983); Osborne et al., Cancer 46:2884-2888 (1980)). The higher the ER amount, the more likely a response. ER is rarely mutated and the

term ER-negative refers to ER level that is below an empirically determined level. Partial hormone response still exists in such cells with low ER levels. It is therefore expected that loss in one of the coactivators
5 (RIZ1) may further reduce hormone response of such cells. Similarly, silencing of a coactivator in ER-positive breast cancers, as shown here for RIZ1, may play a role in the acquisition of hormone resistance phenotypes of these tumors.

10 For hormone sensitive early stage breast cancer, the dual role of RIZ1 as a tumor suppressor and an ER-coactivator/growth promoter raises the issue whether disabling RIZ1 could be beneficial or not to tumor cell proliferation. It appears that retaining RIZ1
15 may be more favorable than disabling it, in view of the reduction of E2-induced tissue growth in RIZ1 deficient mice. This view is also consistent with the finding that RIZ1 silencing was less common in ER-positive versus ER-negative breast cancers.

20 If RIZ1 may confer growth advantage to early stage breast cancers, why is it not so for late stage ER-negative breast cancers, where RIZ1 is commonly silenced? This is expected for a tumor suppressor that also has an ER-dependent growth promoter function. In
25 breast cancers with functional ER, which, upon E2-binding, may turn RIZ1 from a default tumor suppressor into a coactivator/tumor promoter, RIZ1 is often present and may play a role in promoting tumor growth in the presence of E2. In these tumors, RIZ1 may also play a
30 role in keeping tumor growth in check when E2 is absent. In contrast, in ER-negative breast cancers, where a coactivator would be no longer useful and a tumor suppressor would be undesirable, RIZ1 is frequently lost,

which may play a role in promoting the E2-independent growth phenotype of these tumors.

The questions arises as to why most other ER-coactivators are not silenced in hormone resistant breast cancers (Anzick et al., Science 277:965-968 (1997); Kurebayashi et al., Clin. Cancer Res. 6:512-518 (2000); Murphy et al., Cancer Res. 60:6266-6271 (2000); Thenot et al., Mol. Cell. Endocrinol. 156:85-93 (1999)). This is probably because they are not tumor suppressors. It is likely that only a coactivator with tumor suppressor function would represent a preferred target of inactivation during the transition from hormone sensitive to insensitive tumors. During such transition, hormone sensitive tumors will develop mechanisms to grow in the absence of hormonal stimuli or ER function. In these tumor cells that have survived compromised ER, a coactivator may not be useful anymore, and thus there will be no longer a selective pressure to keep it active. Meanwhile, in the absence of the ER switch, the default tumor suppressor function of a coactivator would be on, which would not be favorable to tumors and a selective pressure to inactivate the coactivator may emerge.

These results suggest that RIZ1 possesses intrinsic HMT activity. The RIZ1 specific region (1-200 aa) containing the PR-domain, which is absent in RIZ2, is sufficient for histone H4 methylation in vitro. The PR-domain (residues 30-160) is necessary but insufficient, and requires the less conserved regions (residues 161-200) C-terminal to the PR-domain for HMT activity. The requirement for the region nearby the PR-domain is similar to what has been described for the SET-domain (Rea et al., Nature 406:593-599 (2000)). The activity of the PR domain was impaired by tumor

associated point mutations, consistent with an important role of the activity in tumor suppression functions.

A new methyltransferase catalytic motif was identified, the PBD domain, located at the RIZ1 C-terminus. This domain can methylate H4 in vitro and can physically/functionally cooperate with the PR domain to produce higher HMT activity as well as to broaden substrate specificity. The PBD domain appears not to be significantly related to any proteins in the database. That this domain has HMT activity and can modulate PR activity is consistent with a potential role of this domain in tumor suppression, as indicated by its deletion owing to frameshift mutations in microsatellite unstable cancers.

These data show that a bacteria-expressed 200-residue peptide consisting of the PR-domain region of RIZ1 N-terminus methylated histone H4 while the full-length protein also used H1 and H3 as substrates. The result is consistent with a need for other regions of RIZ1, such as the PBD domain, in modulating the PR domain activity. Indeed, a novel H1 like-protein was methylated only in the presence of both PR and PBD but not by either alone. Alternatively, immunoprecipitated RIZ1 may not be free of RIZ1 bound proteins that could modulate its enzyme activity. RIZ1 may exist as part of a protein complex and the intact nature of such a complex may be required by HMT activity. An example of a HMT complex is the SUV39H1 complex of 650 kDa (Nishioka et al., Genes & Dev. 16:479-489 (2002)). Finally, mammalian specific post-translational modifications of RIZ1 could play a role in substrate specific methylation activities. The latter two possibilities are consistent with the substrate specific regulation of RIZ1 HMT activity by E2

and TPA, and should be an important topic for future investigations.

The results also show that lysine 9 of histone H3 is a major methylation target of RIZ1 protein. RIZ1 protein caused enhanced H3-K9 methylation in vivo and also methylated H3-K9 in vitro. H3-K9 methylation is linked with heterochromatin formation and gene silencing, which in turn, implicates a similar role for RIZ1. Histone H1 and H4 are also methylated by RIZ1 but the role of H1 and H4 methylations in gene transcription remains to be understood. A RIZ1 target residue was identified on H1 in vitro as lysine 25, which is consistent with the known methylation of H1-K25 in vivo (Ohe et al., J. Biochem. (Tokyo) 100:359-368 (1986)).

That E2 can maintain or enhance methylation of one substrate (H1) while decrease methylation of another (H3) suggests a complex mechanism of regulation of RIZ1 activity. The changes in H3 and H1 presumably together contribute to the ligand-dependent RIZ1 activation of ER transactivation functions. That H3-K9 methylation by RIZ1 is inhibited by E2 is consistent with RIZ1 as a coactivator, given that such methylation is linked with gene silencing. The contrasting change in H1 methylation suggests that the role of such methylation is likely to be at least different from, if not the opposite of, that of H3-K9 methylation. H1 phosphorylation is known to be important in transcriptional activation (Bhattacharjee et al., Mol. Cell. Biol. 21:5417-5425 (2001); Dou and Gorovsky, Mol. Cell. 6:225-231 (2000)), although the role of its methylation remains unknown. The finding here that RIZ1 or E2 induced both phosphorylation and methylation of H1 suggests a role for H1 methylation similar to that of H1 phosphorylation. In view of the coactivator function and the previously described

transcriptional repressor function (Xie et al., J. Biol. Chem. 272:26360-26366 (1997)), RIZ1 appears to have a dual role in both transcription activation and repression. The dual role may be mediated in part by
5 methylation of H3-K9 and H1-K25, respectively (Table 4), which may be turned more toward one direction or the other depending on the protein and promoter environment of RIZ1 at particular circumstances. When RIZ1 is recruited by estrogen to activate transcription, the H3
10 methylation function of RIZ1 would be inhibited and the H1 methylation would be maintained or enhanced. The results suggest a model that RIZ1 has a dual role of a tumor suppressor and an ER-coactivator/growth promoter and the same PR-domain is required for both. ER/E2
15 appears to be a switch that can turn RIZ1 from a tumor suppressor into a growth promoter. The dual role of RIZ1 may be mediated by different substrate usage. The H3-K9 methylation/gene repression function of RIZ1 appears to be associated with growth arrest whereas the H1
20 methylation is associated with cell growth, as indicated by the changes of these methylation activities of RIZ1 during terminal differentiation of U937 cells (summarized in Table 4). The tumor suppressor role of RIZ1 is non-tissue specific but the ER-coactivator function is
25 restricted to ER-positive tissues.

Table 4

RIZ1 function ^a	E2 target tissues or ER(+)breast cancer	Non-E2 target tissues or ER(-) breast cancer	Cell proliferation potential	
	+E2	- E2	high	low
5 H1-K25 methylation	++	+	++	+
H1 phosphorylation	+			
Gene activation				
Growth promotion				
10 H3-K9 methylation	+	++	+	++
Gene repression	++			
Growth suppression				

a The functional activity of RIZ1 is indicated as relatively low(+) or high(++)

It seems paradoxical that E2 employs a tumor suppressor for its growth promoting functions. That RIZ1 HMT activity is sensitive to E2 raises the possibility that RIZ1 may have a role as a sensor and breaker to prevent abnormal hormone-independent growth of cells. The presence of RIZ1 in early stage breast cancers may explain the lack of tumor growth in the absence of E2, while its common absence in advanced breast cancers may explain the hormone-independent tumor growth. E2 may serve as a switch to turn off the tumor suppressor function of RIZ1, perhaps by changing target genes and/or HMT activities. Given that the HMT function is required for both tumor suppressor and coactivator functions of RIZ1, E2 must not simply turn off all HMT activities. It is therefore reassuring that E2 can selectively inhibit

RIZ1 HMT activity toward one substrate (H3) while maintain or enhance the activity toward the other (H1).

In summary, the studies described above demonstrate that a HMT (RIZ1) is a target of estrogen and is required for efficient female sex hormone action in vivo. Compromised HMT function of RIZ1 is associated with estrogen resistant breast cancers, and may prove important in the transition to hormone resistance and hormone independent growth during breast cancer progression. By analogy, altered HMT function may also be involved in clinical syndromes characterized by an impaired female sex hormone homeostasis such as osteoporosis, cardiovascular disease, and Alzheimer's disease.

15 EXAMPLE II

This example shows various assays suitable for identifying RIZ1 modulatory compounds and compounds that modulate progesterone receptor activity.

A. *Biochemical assay based on binding of RIZ1 to universal inhibitors of protein methyltransferase activity.*

The best presently known universal inhibitor of PMT is S-Adenosyl-L- or D-homocysteine (SAH, $K_i=5$ to $12 \mu\text{M}$). A less potent inhibitor is Adenosyl-L-ethionine (25 $K_i=170 \mu\text{M}$). SAH mimics the structure of S-adenosyl-L-methionine (SAM) that is the substrate of PMT and the methylgroup donor. RIZ1 is expected to bind to SAH through its PR/PMT motif and its PBD domain.

Multi-well plates are coated with SAH and each well contacted with compounds from a small molecule

chemical compound library. The plates are incubated with purified recombinant protein containing the RIZ1 PR/PMT motif (about 200 amino acids) that has been labeled with a fluorescent probe. After washing away unbound RIZ1
5 protein, the plates are read by a fluorescence detector.

B. Biochemical assay based on binding of RIZ1 to ER or PR

The coactivator function of RIZ1 on ER and PR is dependent upon physical complex formation. Binding of RIZ1 to ER and PR is mediated by the LXXLL motif of RIZ1
10 and is hormone dependent.

Recombinant RIZ1 protein is coated onto multiwell plates and each well contacted with compounds from a small molecule chemical compound library, as well as estrogen or progesterone. The plates are incubated
15 with purified recombinant ER or PR protein that is labeled with a fluorescent probe. After washing away unbound ER or PR protein, the plates are read by a fluorescence detector.

C. Cellular assay based on RIZ1 coactivation of ER or PR

20 Multiwell plates are seeded with CV1 cells and the cells transfected with a plasmid containing a ER responsive promoter linked to a luciferase reporter, a plasmid expressing ER, and a plasmid expressing RIZ1. Each well is contacted with compounds from a small
25 molecule chemical compound library. Two days following transfection, the luciferase activity of each well is assayed and read by a fluorescence detector.

D. Assay to determine or verify the in vivo efficacy of modulators of RIZ1

Once target compounds are obtained through *in vitro* biochemical or cellular high throughput screening, the compounds are tested for *in vivo* efficacy in inhibiting/enhancing the physiological function of RIZ1. Female mice lacking RIZ1 PR/PMT function show decreased response to estrogen and progesterone. Therefore, inhibitors of RIZ1 PR/PMT are expected to decrease estrogen and progesterone responses in mice, while activators of RIZ1 may enhance responses.

Overectomized female mice are treated with estrogen and target compounds for three days. The animals are sacrificed and examined for uterine wet weight and vaginal cornification. In addition, mammary gland growth in response to estrogen and progesterone is determined. 8 week old female mice are ovariectomized at day 0 and treated with E2 (50 µg/day) + P4 (1 µg/day) from day 14-34 (or 21-day releasing hormone pellets containing 0.1 mg of E2 and 10 mg P4; Innovative Research of America). Target compounds are applied together with the hormones. On day 35, the mice are sacrificed and whole mounts prepared and stained. RIZ1 null mutant mice are similarly treated to serve as specificity control for the effects of the target compounds.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. A method of screening for a compound that modulates RIZ histone methyltransferase activity, comprising:
 - 5 a) contacting a RIZ or RIZ fragment having histone methyltransferase activity with one or more candidate compounds; and
 - b) determining histone methyltransferase activity of said contacted RIZ or RIZ fragment,
- 10 wherein a compound that modulates RIZ histone methyltransferase activity is identified.
2. The method of claim 1, wherein said RIZ is RIZ1.
3. The method of claim 1, wherein said RIZ is RIZ2.
4. The method of claim 1, wherein said RIZ fragment
15 comprises amino acids 1-200 of human RIZ1.
5. The method of claim 1, wherein said RIZ fragment comprises amino acids 1-332 of human RIZ1.
6. The method of claim 1, wherein said RIZ fragment comprises amino acids 1514-1680 of human RIZ1.
- 20 7. The method of claim 1, wherein histone H1 methyltransferase activity is determined.
8. The method of claim 1, wherein histone H3 methyltransferase activity is determined.
9. The method of claim 1, wherein histone H4
25 methyltransferase activity is determined.

10. A method of screening for a compound that modulates progesterone receptor activity, comprising:

- a) providing a RIZ1 modulatory compound;
 - b) determining the ability of said RIZ1 modulatory
- 5 compound to modulate progesterone receptor activity, wherein a compound that modulates progesterone receptor activity is identified.

11. The method of claim 10, wherein step a) comprises:

- 10 i) contacting RIZ1 with one or more candidate compounds;
 - ii) determining the ability of said candidate compound to selectively bind RIZ1; and
 - iii) providing a compound from step ii) that
- 15 selectively binds RIZ1.

12. The method of claim 10, wherein step a) comprises:

- i) contacting RIZ1 with one or more candidate compounds under conditions wherein RIZ1 selectively binds
- 20 a RIZ1-binding partner;
- ii) determining the ability of said candidate compound to modulate said selective binding between RIZ1 and said RIZ1-binding partner; and
 - iii) providing a compound from step ii) that
- 25 modulates said selective binding.

13. The method of claim 10, wherein step a) comprises:

- i) contacting RIZ1 with one or more candidate compounds under conditions wherein RIZ1 exhibits a
5 functional activity;
- ii) determining the ability of said candidate compound to modulate said functional activity; and
- iii) providing a compound from step ii) that modulates said functional activity.

10 14. The method of claim 13, wherein said functional activity is histone methyltransferase activity.

15 15. The method of claim 10, wherein step b) comprises determining the ability of said RIZ1 modulatory compound to modulate progesterone receptor-mediated transcription.

16. The method of claim 10, wherein step b) comprises determining the ability of said RIZ1 modulatory compound to modulate progesterone-dependent mammapoiesis.

20 17. The method of claim 10, wherein step b) comprises determining the ability of said RIZ1 modulatory compound to modulate progesterone-dependent uterine development.

25 18. A method of identifying an individual with an estrogen receptor positive (ER+) tumor having a reduced likelihood of responding to endocrine therapy, comprising determining the RIZ1 status of said tumor, wherein an abnormal RIZ1 status identifies said individual as an individual with a reduced likelihood of responding to endocrine therapy.

19. The method of claim 18, wherein abnormal RIZ1 status is evidenced by RIZ1 promoter methylation.

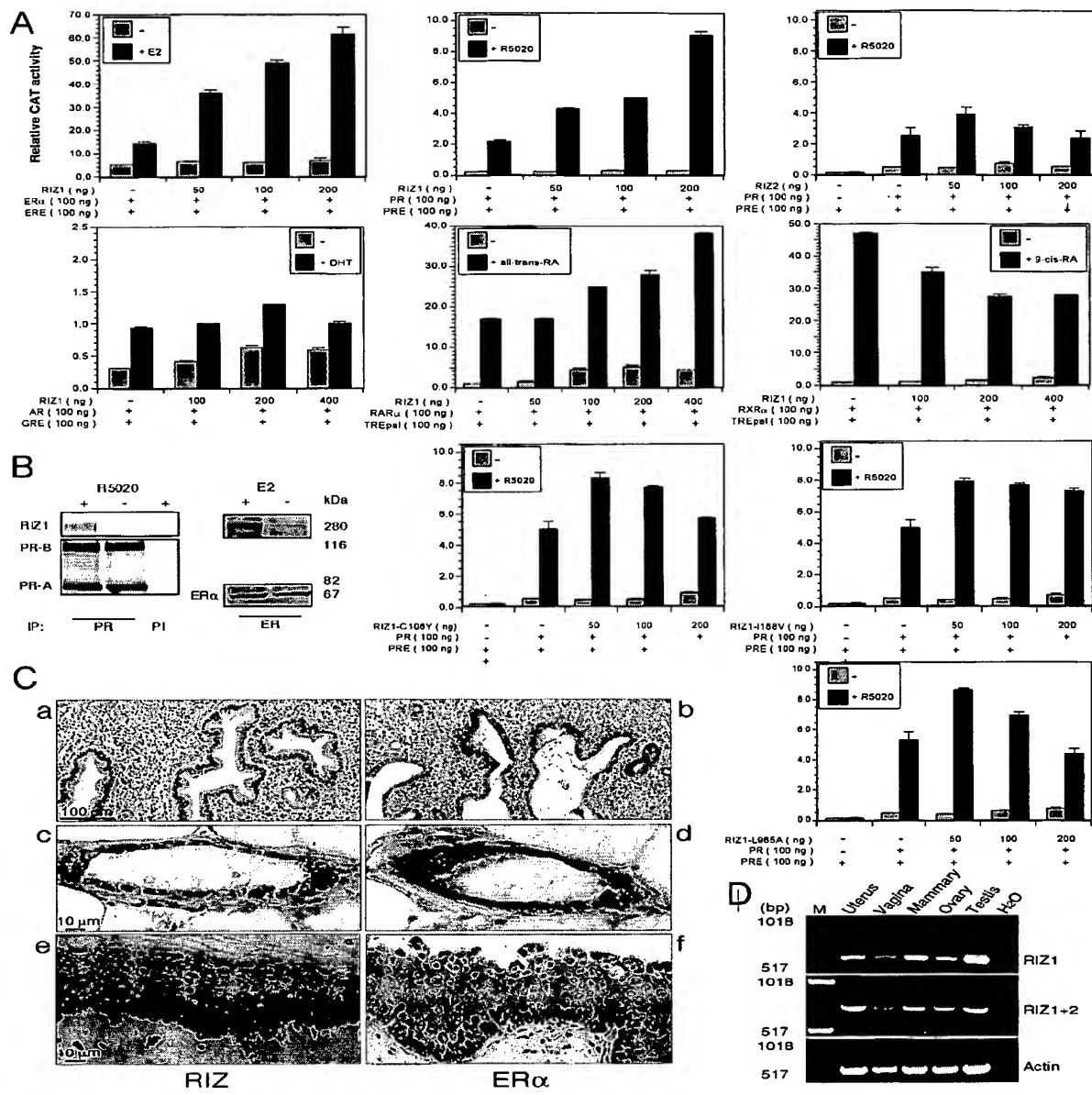
20. The method of claim 18, wherein abnormal RIZ1 status is evidenced by decreased RIZ1 protein expression.

5 21. The method of claim 18, wherein abnormal RIZ1 status is evidenced by decreased RIZ1 mRNA expression.

22. The method of claim 18, wherein abnormal RIZ1 status is evidenced by a mutation in the RIZ1 gene.

23. The method of claim 18, wherein abnormal RIZ1
10 status is evidenced by altered RIZ1 histone methyltransferase activity.

24. The method of claim 23, wherein said altered RIZ1 histone methyltransferase activity is increased H1 methylation or decreased H3 methylation.



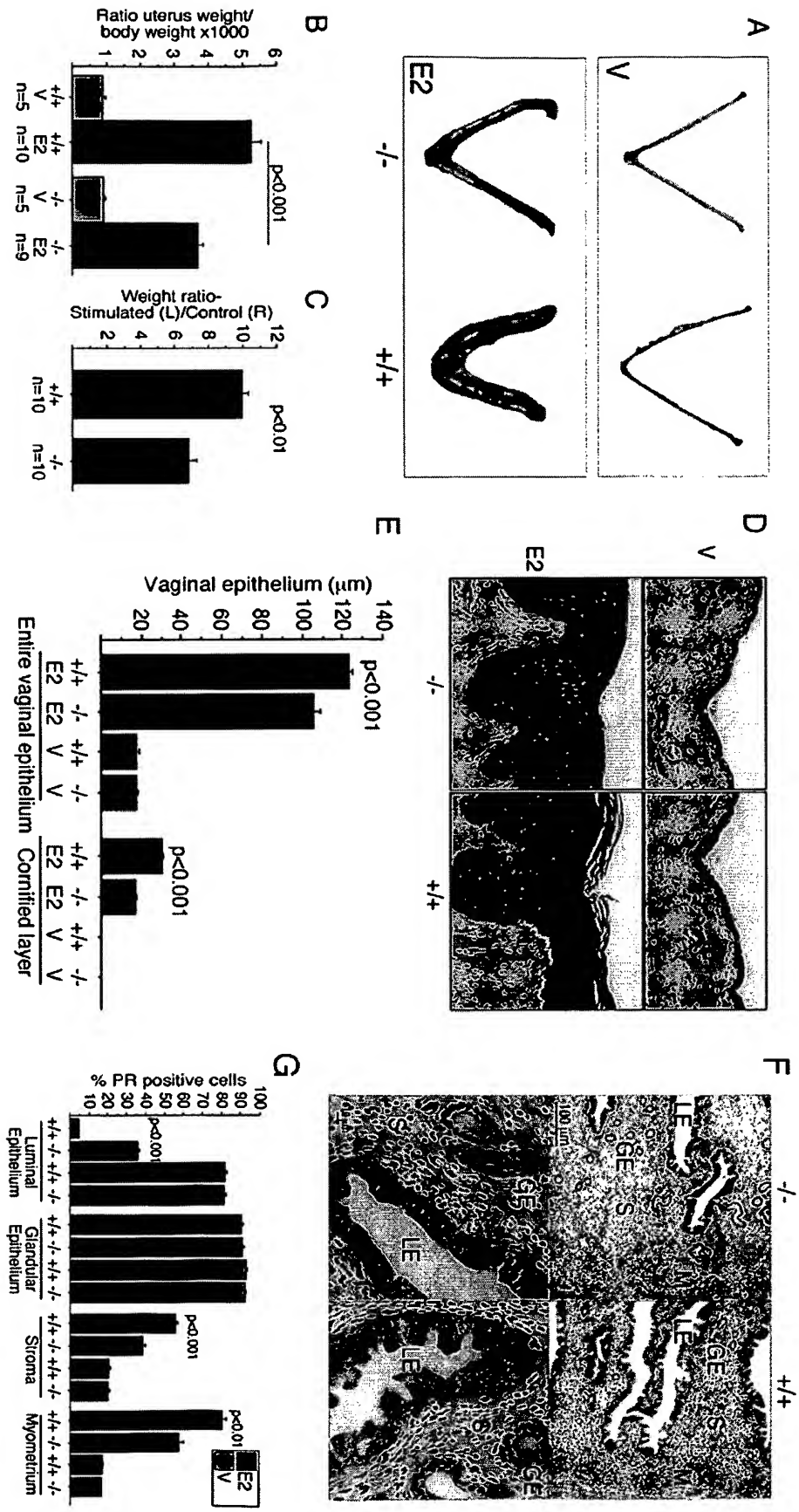


FIGURE 2

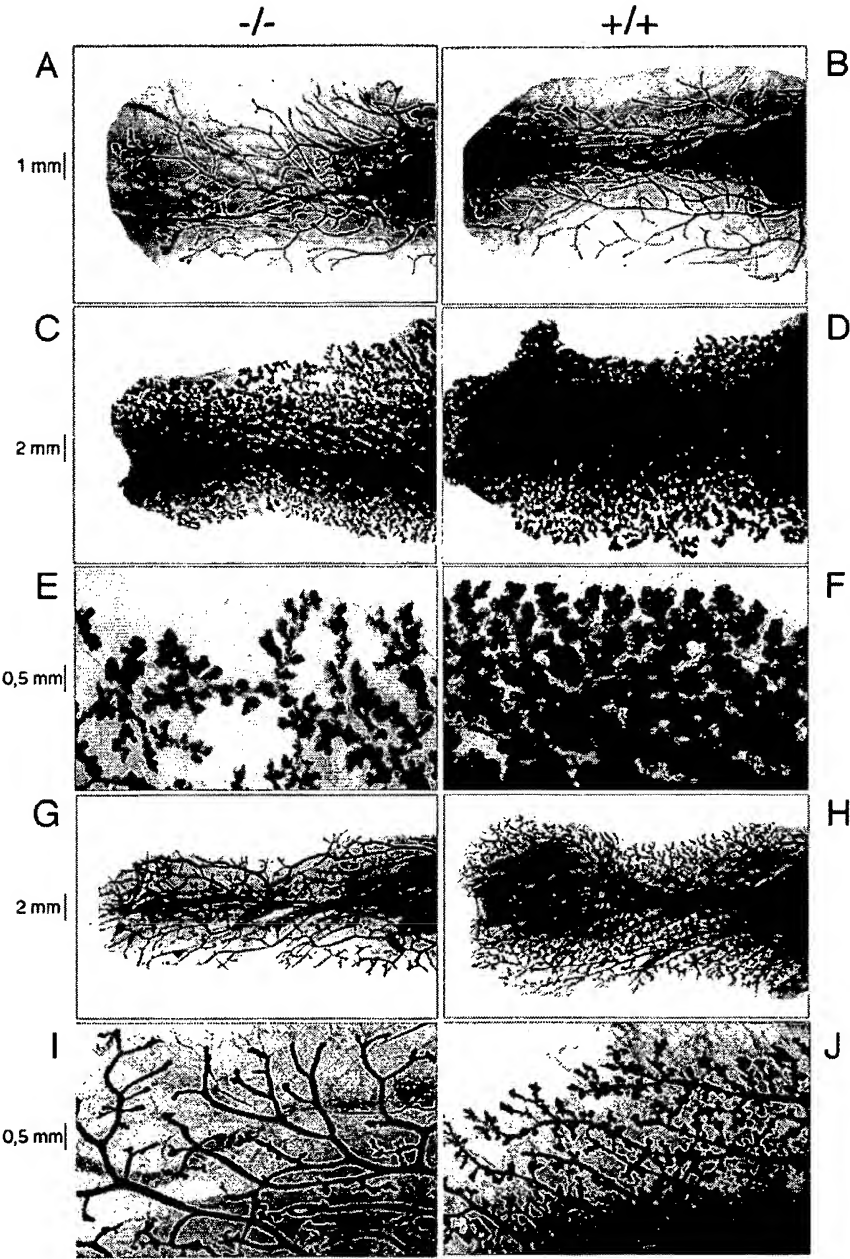


FIGURE 3

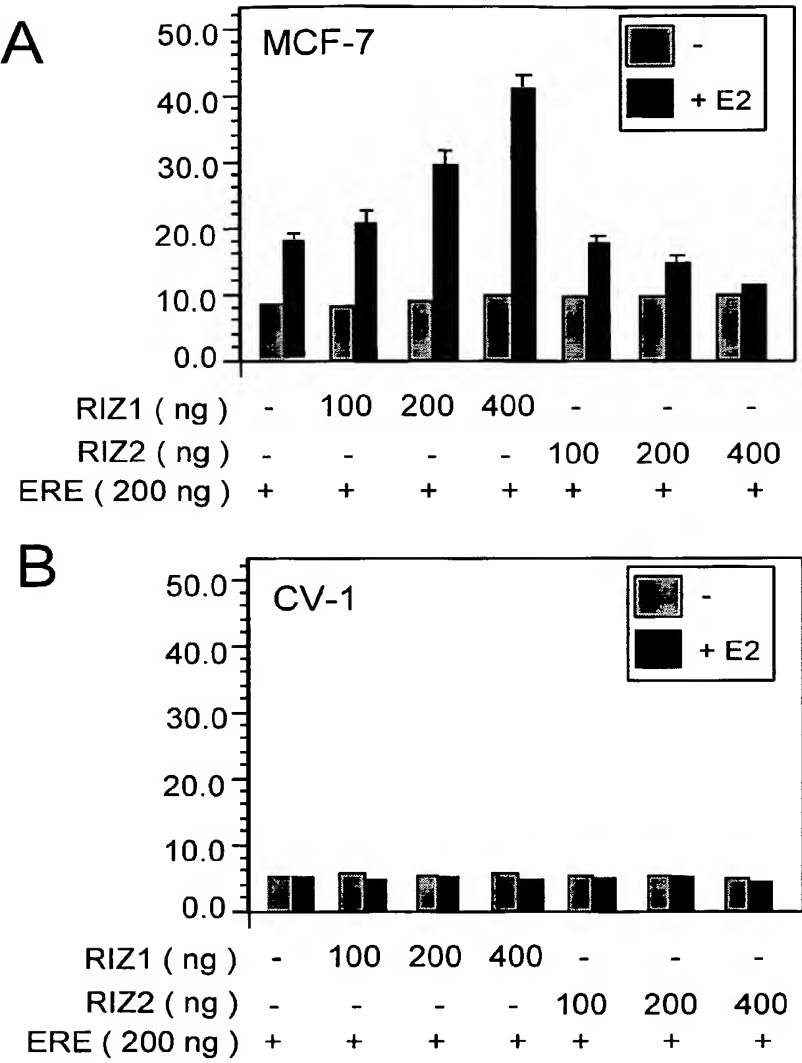


FIGURE 4

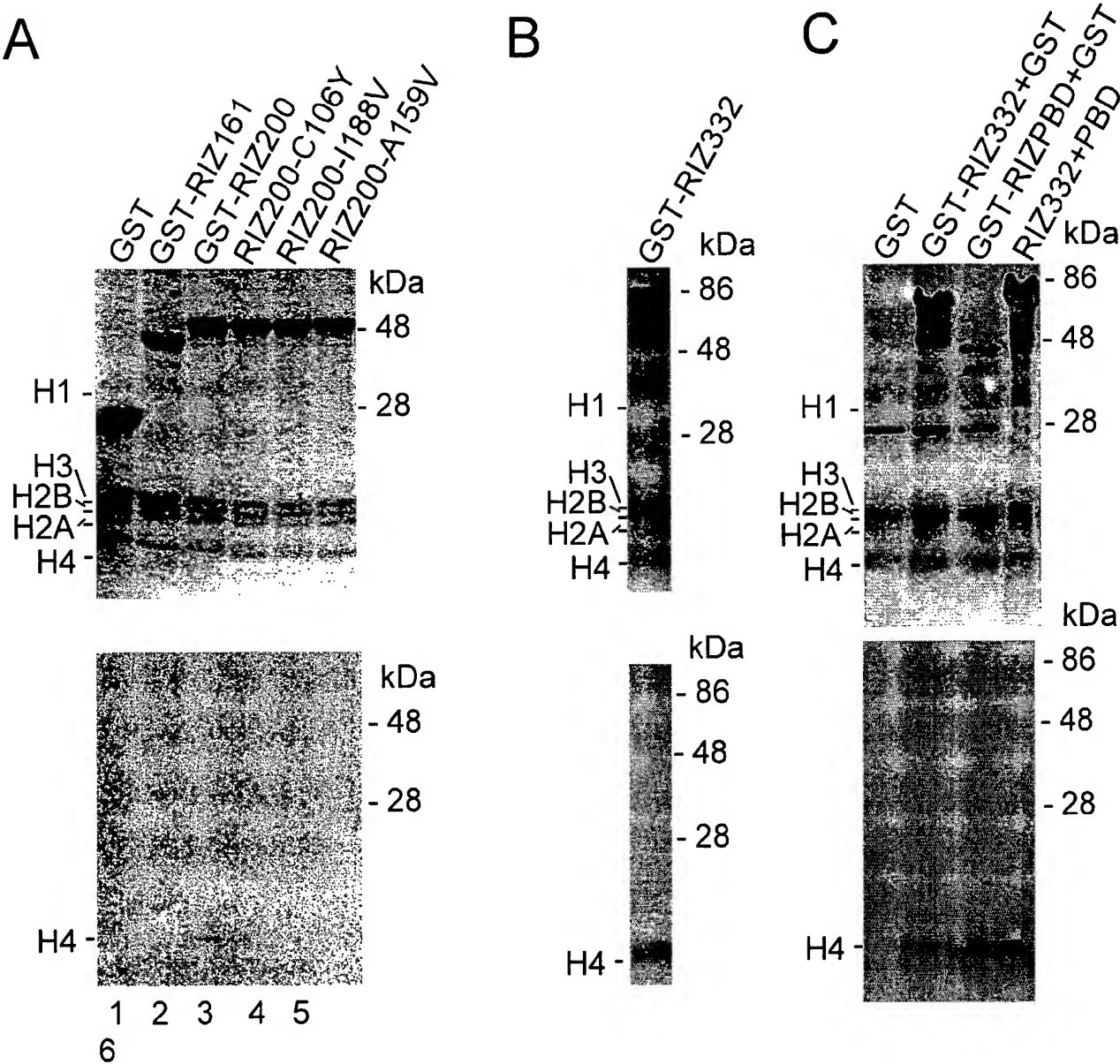


FIGURE 5

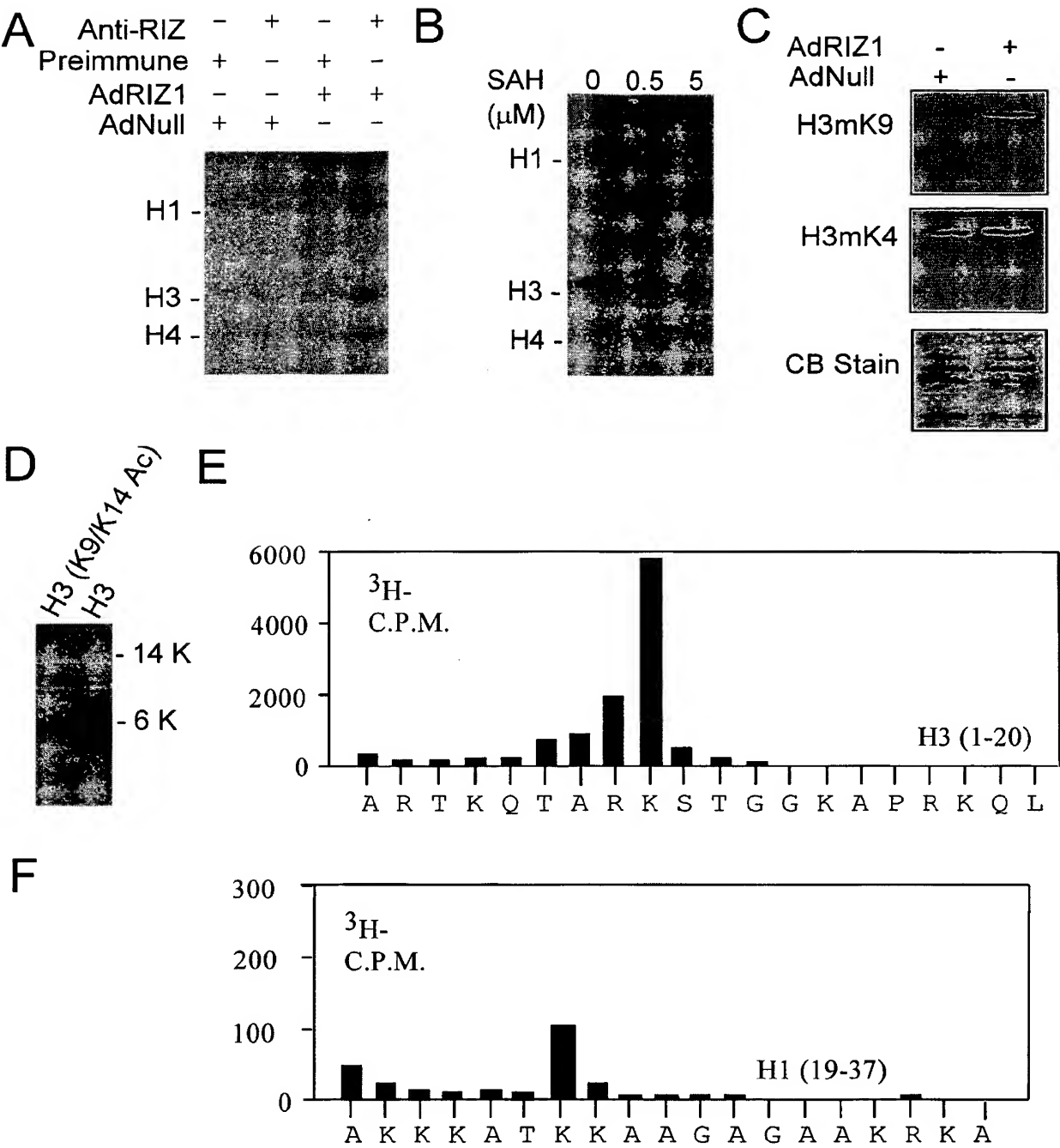


FIGURE 6

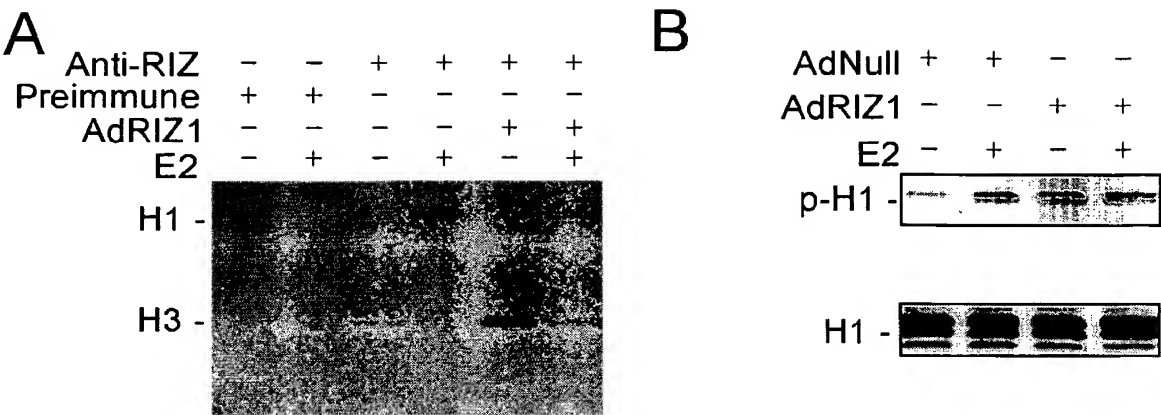


FIGURE 7

SEQUENCE LISTING

<110> The Burnham Institute
Huang, Shi

<120> Screening, Diagnostic and Therapeutic
Methods Relating To RIZ

<130> FP-LJ 5225

<150> US 60/290,481

<151> 2001-05-11

<160> 19

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 1719

<212> PRT

<213> Homo sapiens

<400> 1

Met	Asn	Gln	Asn	Thr	Thr	Glu	Pro	Val	Ala	Ala	Thr	Glu	Thr	Leu	Ala
1			5						10					15	
Glu	Val	Pro	Glu	His	Val	Leu	Arg	Gly	Leu	Pro	Glu	Glu	Val	Arg	Leu
			20					25					30		
Phe	Pro	Ser	Ala	Val	Asp	Lys	Thr	Arg	Ile	Gly	Val	Trp	Ala	Thr	Lys
		35				40						45			
Pro	Ile	Leu	Lys	Gly	Lys	Lys	Phe	Gly	Pro	Phe	Val	Gly	Asp	Lys	Lys
	50					55					60				
Lys	Arg	Ser	Gln	Val	Lys	Asn	Asn	Val	Tyr	Met	Trp	Glu	Val	Tyr	Tyr
65					70					75				80	
Pro	Asn	Leu	Gly	Trp	Met	Cys	Ile	Asp	Ala	Thr	Asp	Pro	Glu	Lys	Gly
				85					90					95	
Asn	Trp	Leu	Arg	Tyr	Val	Asn	Trp	Ala	Cys	Ser	Gly	Glu	Glu	Gln	Asn
			100					105						110	
Leu	Phe	Pro	Leu	Glu	Ile	Asn	Arg	Ala	Ile	Tyr	Tyr	Lys	Thr	Leu	Lys
		115					120					125			
Pro	Ile	Ala	Pro	Gly	Glu	Glu	Leu	Leu	Val	Trp	Tyr	Asn	Gly	Glu	Asp
	130					135					140				
Asn	Pro	Glu	Ile	Ala	Ala	Ala	Ile	Glu	Glu	Glu	Arg	Ala	Ser	Ala	Arg
145					150					155				160	
Ser	Lys	Arg	Ser	Ser	Pro	Lys	Ser	Arg	Lys	Gly	Lys	Lys	Lys	Ser	Gln
				165					170					175	
Glu	Asn	Lys	Asn	Lys	Gly	Asn	Lys	Ile	Gln	Asp	Ile	Gln	Leu	Lys	Thr
			180				185					190			
Ser	Glu	Pro	Asp	Phe	Thr	Ser	Ala	Asn	Met	Arg	Asp	Ser	Ala	Glu	Gly
		195					200					205			
Pro	Lys	Glu	Asp	Glu	Glu	Lys	Pro	Ser	Ala	Ser	Ala	Leu	Glu	Gln	Pro
	210					215					220				
Ala	Thr	Leu	Gln	Glu	Val	Ala	Ser	Gln	Glu	Val	Pro	Pro	Glu	Leu	Ala
225					230				235					240	

Thr Pro Ala Pro Ala Trp Glu Pro Gln Pro Glu Pro Asp Glu Arg Leu
 245 250 255
 Glu Ala Ala Ala Cys Glu Val Asn Asp Leu Gly Glu Glu Glu Glu
 260 265 270
 Glu Glu Glu Glu Asp Glu Glu Glu Glu Asp Asp Asp Asp Glu
 275 280 285
 Leu Glu Asp Glu Gly Glu Glu Glu Ala Ser Met Pro Asn Glu Asn Ser
 290 295 300
 Val Lys Glu Pro Glu Ile Arg Cys Asp Glu Lys Pro Glu Asp Leu Leu
 305 310 315 320
 Glu Glu Pro Lys Thr Thr Ser Glu Glu Thr Leu Glu Asp Cys Ser Glu
 325 330 335
 Val Thr Pro Ala Met Gln Ile Pro Arg Thr Lys Glu Glu Ala Asn Gly
 340 345 350
 Asp Val Phe Glu Thr Phe Met Phe Pro Cys Gln His Cys Glu Arg Lys
 355 360 365
 Phe Thr Thr Lys Gln Gly Leu Glu Arg His Met His Ile His Ile Ser
 370 375 380
 Thr Val Asn His Ala Phe Lys Cys Lys Tyr Cys Gly Lys Ala Phe Gly
 385 390 395 400
 Thr Gln Ile Asn Arg Arg Arg His Glu Arg Arg His Glu Ala Gly Leu
 405 410 415
 Lys Arg Lys Pro Ser Gln Thr Leu Gln Pro Ser Glu Asp Leu Ala Asp
 420 425 430
 Gly Lys Ala Ser Gly Glu Asn Val Ala Ser Lys Asp Asp Ser Ser Pro
 435 440 445
 Pro Ser Leu Gly Pro Asp Cys Leu Ile Met Asn Ser Glu Lys Ala Ser
 450 455 460
 Gln Asp Thr Ile Asn Ser Ser Val Val Glu Glu Asn Gly Glu Val Lys
 465 470 475 480
 Glu Leu His Pro Cys Lys Tyr Cys Lys Lys Val Phe Gly Thr His Thr
 485 490 495
 Asn Met Arg Arg His Gln Arg Arg Val His Glu Arg His Leu Ile Pro
 500 505 510
 Lys Gly Val Arg Arg Lys Gly Gly Leu Glu Glu Pro Gln Pro Pro Ala
 515 520 525
 Glu Gln Ala Gln Ala Thr Gln Asn Val Tyr Val Pro Ser Thr Glu Pro
 530 535 540
 Glu Glu Glu Gly Glu Ala Asp Asp Val Tyr Ile Met Asp Ile Ser Ser
 545 550 555 560
 Asn Ile Ser Glu Asn Leu Asn Tyr Tyr Ile Asp Gly Lys Ile Gln Thr
 565 570 575
 Asn Asn Asn Thr Ser Asn Cys Asp Val Ile Glu Met Glu Ser Ala Ser
 580 585 590
 Ala Asp Leu Tyr Gly Ile Asn Cys Leu Leu Thr Pro Val Thr Val Glu
 595 600 605
 Ile Thr Gln Asn Ile Lys Thr Thr Gln Val Pro Val Thr Glu Asp Leu
 610 615 620
 Pro Lys Glu Pro Leu Gly Ser Thr Asn Ser Glu Ala Lys Lys Arg Arg
 625 630 635 640
 Thr Ala Ser Pro Pro Ala Leu Pro Lys Ile Lys Ala Glu Thr Asp Ser
 645 650 655
 Asp Pro Met Val Pro Ser Cys Ser Leu Ser Leu Pro Leu Ser Ile Ser
 660 665 670
 Thr Thr Glu Ala Val Ser Phe His Lys Glu Lys Ser Val Tyr Leu Ser

675	680	685
Ser Lys Leu Lys Gln Leu Leu Gln Thr Gln Asp Lys Leu Thr Pro Pro		
690	695	700
Ala Gly Ile Ser Ala Thr Glu Ile Ala Lys Leu Gly Pro Val Cys Val		
705	710	715
Ser Ala Pro Ala Ser Met Leu Pro Val Thr Ser Ser Arg Phe Lys Arg		
725	730	735
Arg Thr Ser Ser Pro Pro Ser Ser Pro Gln His Ser Pro Ala Leu Arg		
740	745	750
Asp Phe Gly Lys Pro Ser Asp Gly Lys Ala Ala Trp Thr Asp Ala Gly		
755	760	765
Leu Thr Ser Lys Lys Ser Lys Leu Glu Ser His Ser Asp Ser Pro Ala		
770	775	780
Trp Ser Leu Ser Gly Arg Asp Glu Arg Glu Thr Val Ser Pro Pro Cys		
785	790	795
Phe Asp Glu Tyr Lys Met Ser Lys Glu Trp Thr Ala Ser Ser Ala Phe		
805	810	815
Ser Ser Val Cys Asn Gln Gln Pro Leu Asp Leu Ser Ser Gly Val Lys		
820	825	830
Gln Lys Ala Glu Gly Thr Gly Lys Thr Pro Val Gln Trp Glu Ser Val		
835	840	845
Leu Asp Leu Ser Val His Lys Lys His Cys Ser Asp Ser Glu Gly Lys		
850	855	860
Glu Phe Lys Glu Ser His Ser Val Gln Pro Thr Cys Ser Ala Val Lys		
865	870	875
Lys Arg Lys Pro Thr Thr Cys Met Leu Gln Lys Val Leu Leu Asn Glu		
885	890	895
Tyr Asn Gly Ile Asp Leu Pro Val Glu Asn Pro Ala Asp Gly Thr Arg		
900	905	910
Ser Pro Ser Pro Cys Lys Ser Leu Glu Ala Gln Pro Asp Pro Asp Leu		
915	920	925
Gly Pro Gly Ser Gly Phe Pro Ala Pro Thr Val Glu Ser Thr Pro Asp		
930	935	940
Val Cys Pro Ser Ser Pro Ala Leu Gln Thr Pro Ser Leu Ser Ser Gly		
945	950	955
Gln Leu Pro Pro Leu Leu Ile Pro Thr Asp Pro Ser Ser Pro Pro Pro		
965	970	975
Cys Pro Pro Val Leu Thr Val Ala Thr Pro Pro Pro Pro Leu Leu Pro		
980	985	990
Thr Val Pro Leu Pro Ala Pro Ser Ser Ser Ala Ser Pro His Pro Cys		
995	1000	1005
Pro Ser Pro Leu Ser Asn Ala Thr Ala Gln Ser Pro Leu Pro Ile Leu		
1010	1015	1020
Ser Pro Thr Val Ser Pro Ser Pro Ser Pro Ile Pro Pro Val Glu Pro		
1025	1030	1035
Leu Met Ser Ala Ala Ser Pro Gly Pro Pro Thr Leu Ser Ser Ser Ser		
1045	1050	1055
Ser Ser Ser Ser Ser Ser Ser Phe Ser Ser Ser Ser Ser Ser Ser		
1060	1065	1070
Ser Pro Ser Pro Pro Pro Leu Ser Ala Ile Ser Ser Val Val Ser Ser		
1075	1080	1085
Gly Asp Asn Leu Glu Ala Ser Leu Pro Met Ile Ser Phe Lys Gln Glu		
1090	1095	1100
Glu Leu Glu Asn Glu Gly Leu Lys Pro Arg Glu Glu Pro Gln Ser Ala		
1105	1110	1115
		1120

Ala Glu Gln Asp Val Val Val Gln Glu Thr Phe Asn Lys Asn Phe Val
 1125 1130 1135
 Cys Asn Val Cys Glu Ser Pro Phe Leu Ser Ile Lys Asp Leu Thr Lys
 1140 1145 1150
 His Leu Ser Ile His Ala Glu Glu Trp Pro Phe Lys Cys Glu Phe Cys
 1155 1160 1165
 Val Gln Leu Phe Lys Asp Lys Thr Asp Leu Ser Glu His Arg Phe Leu
 1170 1175 1180
 Leu His Gly Val Gly Asn Ile Phe Val Cys Ser Val Cys Lys Lys Glu
 1185 1190 1195 1200
 Phe Ala Phe Leu Cys Asn Leu Gln Gln His Gln Arg Asp Leu His Pro
 1205 1210 1215
 Asp Lys Val Cys Thr His His Glu Phe Glu Ser Gly Thr Leu Arg Pro
 1220 1225 1230
 Gln Asn Phe Thr Asp Pro Ser Lys Ala His Val Glu His Met Gln Ser
 1235 1240 1245
 Leu Pro Glu Asp Pro Leu Glu Thr Ser Lys Glu Glu Glu Leu Asn
 1250 1255 1260
 Asp Ser Ser Glu Glu Leu Tyr Thr Thr Ile Lys Ile Met Ala Ser Gly
 1265 1270 1275 1280
 Ile Lys Thr Lys Asp Pro Asp Val Arg Leu Gly Leu Asn Gln His Tyr
 1285 1290 1295
 Pro Ser Phe Lys Pro Pro Pro Phe Gln Tyr His His Arg Asn Pro Met
 1300 1305 1310
 Gly Ile Gly Val Thr Ala Thr Asn Phe Thr Thr His Asn Ile Pro Gln
 1315 1320 1325
 Thr Phe Thr Thr Ala Ile Arg Cys Thr Lys Cys Gly Lys Gly Val Asp
 1330 1335 1340
 Asn Met Pro Glu Leu His Lys His Ile Leu Ala Cys Ala Ser Ala Ser
 1345 1350 1355 1360
 Asp Lys Lys Arg Tyr Thr Pro Lys Lys Asn Pro Val Pro Leu Lys Gln
 1365 1370 1375
 Thr Val Gln Pro Lys Asn Gly Val Val Val Leu Asp Asn Ser Gly Lys
 1380 1385 1390
 Asn Ala Phe Arg Arg Met Gly Gln Pro Lys Arg Leu Asn Phe Ser Val
 1395 1400 1405
 Glu Leu Ser Lys Met Ser Ser Asn Lys Leu Lys Leu Asn Ala Leu Lys
 1410 1415 1420
 Lys Lys Asn Gln Leu Val Gln Lys Ala Ile Leu Gln Lys Asn Lys Ser
 1425 1430 1435 1440
 Ala Lys Gln Lys Ala Asp Leu Lys Asn Ala Cys Glu Ser Ser Ser His
 1445 1450 1455
 Ile Cys Pro Tyr Cys Asn Arg Glu Phe Thr Tyr Ile Gly Ser Leu Asn
 1460 1465 1470
 Lys His Ala Ala Phe Ser Cys Pro Lys Lys Pro Leu Ser Pro Pro Lys
 1475 1480 1485
 Lys Lys Val Ser His Ser Ser Lys Lys Gly Gly His Ser Ser Pro Ala
 1490 1495 1500
 Ser Ser Asp Lys Asn Ser Asn Ser Asn His Arg Arg Arg Thr Ala Asp
 1505 1510 1515 1520
 Ala Glu Ile Lys Met Gln Ser Met Gln Thr Pro Leu Gly Lys Thr Arg
 1525 1530 1535
 Ala Arg Ser Ser Gly Pro Thr Gln Val Pro Leu Pro Ser Ser Ser Phe
 1540 1545 1550
 Arg Ser Lys Gln Asn Val Lys Phe Ala Ala Ser Val Lys Ser Lys Lys

1555 1560 1565
 Pro Ser Ser Ser Ser Leu Arg Asn Ser Ser Pro Ile Arg Met Ala Lys
 1570 1575 1580
 Ile Thr His Val Glu Gly Lys Lys Pro Lys Ala Val Ala Lys Asn His
 1585 1590 1595 1600
 Ser Ala Gln Leu Ser Ser Lys Thr Ser Arg Ser Leu His Val Arg Val
 1605 1610 1615
 Gln Lys Ser Lys Ala Val Leu Gln Ser Lys Ser Thr Leu Ala Ser Lys
 1620 1625 1630
 Lys Arg Thr Asp Arg Phe Asn Ile Lys Ser Arg Glu Arg Ser Gly Gly
 1635 1640 1645
 Pro Val Thr Arg Ser Leu Gln Leu Ala Ala Ala Asp Leu Ser Glu
 1650 1655 1660
 Asn Lys Arg Glu Asp Gly Ser Ala Lys Gln Glu Leu Lys Asp Phe Ser
 1665 1670 1675 1680
 Tyr Ser Leu Arg Leu Ala Ser Arg Cys Ser Pro Pro Ala Ala Pro Tyr
 1685 1690 1695
 Ile Thr Arg Gln Tyr Arg Lys Val Lys Ala Pro Ala Ala Ala Gln Phe
 1700 1705 1710
 Gln Gly Pro Phe Phe Lys Glu
 1715

<210> 2
 <211> 1518
 <212> PRT
 <213> Homo sapiens

<400> 2
 Met Arg Asp Ser Ala Glu Gly Pro Lys Glu Asp Glu Glu Lys Pro Ser
 1 5 10 15
 Ala Ser Ala Leu Glu Gln Pro Ala Thr Leu Gln Glu Val Ala Ser Gln
 20 25 30
 Glu Val Pro Pro Glu Leu Ala Thr Pro Ala Pro Ala Trp Glu Pro Gln
 35 40 45
 Pro Glu Pro Asp Glu Arg Leu Glu Ala Ala Ala Cys Glu Val Asn Asp
 50 55 60
 Leu Gly Glu Glu Glu Glu Glu Glu Glu Glu Glu Asp Glu Glu Glu Glu
 65 70 75 80
 Glu Asp Asp Asp Asp Asp Glu Leu Glu Asp Glu Gly Glu Glu Glu Ala
 85 90 95
 Ser Met Pro Asn Glu Asn Ser Val Lys Glu Pro Glu Ile Arg Cys Asp
 100 105 110
 Glu Lys Pro Glu Asp Leu Leu Glu Glu Pro Lys Thr Thr Ser Glu Glu
 115 120 125
 Thr Leu Glu Asp Cys Ser Glu Val Thr Pro Ala Met Gln Ile Pro Arg
 130 135 140
 Thr Lys Glu Glu Ala Asn Gly Asp Val Phe Glu Thr Phe Met Phe Pro
 145 150 155 160
 Cys Gln His Cys Glu Arg Lys Phe Thr Thr Lys Gln Gly Leu Glu Arg
 165 170 175
 His Met His Ile His Ile Ser Thr Val Asn His Ala Phe Lys Cys Lys
 180 185 190
 Tyr Cys Gly Lys Ala Phe Gly Thr Gln Ile Asn Arg Arg Arg His Glu
 195 200 205

Arg	Arg	His	Glu	Ala	Gly	Leu	Lys	Arg	Lys	Pro	Ser	Gln	Thr	Leu	Gln
210						215					220				
Pro	Ser	Glu	Asp	Leu	Ala	Asp	Gly	Lys	Ala	Ser	Gly	Glu	Asn	Val	Ala
225					230					235					240
Ser	Lys	Asp	Asp	Ser	Ser	Pro	Pro	Ser	Leu	Gly	Pro	Asp	Cys	Leu	Ile
				245					250					255	
Met	Asn	Ser	Glu	Lys	Ala	Ser	Gln	Asp	Thr	Ile	Asn	Ser	Ser	Val	Val
			260					265					270		
Glu	Glu	Asn	Gly	Glu	Val	Lys	Glu	Leu	His	Pro	Cys	Lys	Tyr	Cys	Lys
		275					280					285			
Lys	Val	Phe	Gly	Thr	His	Thr	Asn	Met	Arg	Arg	His	Gln	Arg	Arg	Val
	290					295					300				
His	Glu	Arg	His	Leu	Ile	Pro	Lys	Gly	Val	Arg	Arg	Lys	Gly	Gly	Leu
305					310					315					320
Glu	Glu	Pro	Gln	Pro	Pro	Ala	Glu	Gln	Ala	Gln	Ala	Thr	Gln	Asn	Val
				325					330					335	
Tyr	Val	Pro	Ser	Thr	Glu	Pro	Glu	Glu	Glu	Gly	Glu	Ala	Asp	Asp	Val
			340				345						350		
Tyr	Ile	Met	Asp	Ile	Ser	Ser	Asn	Ile	Ser	Glu	Asn	Leu	Asn	Tyr	Tyr
	355						360					365			
Ile	Asp	Gly	Lys	Ile	Gln	Thr	Asn	Asn	Asn	Thr	Ser	Asn	Cys	Asp	Val
	370					375					380				
Ile	Glu	Met	Glu	Ser	Ala	Ser	Ala	Asp	Leu	Tyr	Gly	Ile	Asn	Cys	Leu
385					390					395					400
Leu	Thr	Pro	Val	Thr	Val	Glu	Ile	Thr	Gln	Asn	Ile	Lys	Thr	Thr	Gln
				405					410					415	
Val	Pro	Val	Thr	Glu	Asp	Leu	Pro	Lys	Glu	Pro	Leu	Gly	Ser	Thr	Asn
			420					425					430		
Ser	Glu	Ala	Lys	Lys	Arg	Arg	Thr	Ala	Ser	Pro	Pro	Ala	Leu	Pro	Lys
		435					440					445			
Ile	Lys	Ala	Glu	Thr	Asp	Ser	Asp	Pro	Met	Val	Pro	Ser	Cys	Ser	Leu
	450					455					460				
Ser	Leu	Pro	Leu	Ser	Ile	Ser	Thr	Thr	Glu	Ala	Val	Ser	Phe	His	Lys
465					470					475					480
Glu	Lys	Ser	Val	Tyr	Leu	Ser	Ser	Lys	Leu	Lys	Gln	Leu	Leu	Gln	Thr
				485					490					495	
Gln	Asp	Lys	Leu	Thr	Pro	Pro	Ala	Gly	Ile	Ser	Ala	Thr	Glu	Ile	Ala
			500					505					510		
Lys	Leu	Gly	Pro	Val	Cys	Val	Ser	Ala	Pro	Ala	Ser	Met	Leu	Pro	Val
		515						520				525			
Thr	Ser	Ser	Arg	Phe	Lys	Arg	Arg	Thr	Ser	Ser	Pro	Pro	Ser	Ser	Pro
	530					535					540				
Gln	His	Ser	Pro	Ala	Leu	Arg	Asp	Phe	Gly	Lys	Pro	Ser	Asp	Gly	Lys
545					550					555					560
Ala	Ala	Trp	Thr	Asp	Ala	Gly	Leu	Thr	Ser	Lys	Lys	Ser	Lys	Leu	Glu
				565					570					575	
Ser	His	Ser	Asp	Ser	Pro	Ala	Trp	Ser	Leu	Ser	Gly	Arg	Asp	Glu	Arg
			580					585				590			
Glu	Thr	Val	Ser	Pro	Pro	Cys	Phe	Asp	Glu	Tyr	Lys	Met	Ser	Lys	Glu
		595					600					605			
Trp	Thr	Ala	Ser	Ser	Ala	Phe	Ser	Ser	Val	Cys	Asn	Gln	Gln	Pro	Leu
	610					615					620				
Asp	Leu	Ser	Ser	Gly	Val	Lys	Gln	Lys	Ala	Glu	Gly	Thr	Gly	Lys	Thr
625					630					635					640
Pro	Val	Gln	Trp	Glu	Ser	Val	Leu	Asp	Leu	Ser	Val	His	Lys	Lys	His

- 7 -

Leu Gly Leu Asn Gln His Tyr Pro Ser Phe Lys Pro Pro Pro Phe Gln
 1090 1095 1100
 Tyr His His Arg Asn Pro Met Gly Ile Gly Val Thr Ala Thr Asn Phe
 1105 1110 1115 1120
 Thr Thr His Asn Ile Pro Gln Thr Phe Thr Thr Ala Ile Arg Cys Thr
 1125 1130 1135
 Lys Cys Gly Lys Gly Val Asp Asn Met Pro Glu Leu His Lys His Ile
 1140 1145 1150
 Leu Ala Cys Ala Ser Ala Ser Asp Lys Lys Arg Tyr Thr Pro Lys Lys
 1155 1160 1165
 Asn Pro Val Pro Leu Lys Gln Thr Val Gln Pro Lys Asn Gly Val Val
 1170 1175 1180
 Val Leu Asp Asn Ser Gly Lys Asn Ala Phe Arg Arg Met Gly Gln Pro
 1185 1190 1195 1200
 Lys Arg Leu Asn Phe Ser Val Glu Leu Ser Lys Met Ser Ser Asn Lys
 1205 1210 1215
 Leu Lys Leu Asn Ala Leu Lys Lys Lys Asn Gln Leu Val Gln Lys Ala
 1220 1225 1230
 Ile Leu Gln Lys Asn Lys Ser Ala Lys Gln Lys Ala Asp Leu Lys Asn
 1235 1240 1245
 Ala Cys Glu Ser Ser Ser His Ile Cys Pro Tyr Cys Asn Arg Glu Phe
 1250 1255 1260
 Thr Tyr Ile Gly Ser Leu Asn Lys His Ala Ala Phe Ser Cys Pro Lys
 1265 1270 1275 1280
 Lys Pro Leu Ser Pro Pro Lys Lys Lys Val Ser His Ser Ser Lys Lys
 1285 1290 1295
 Gly Gly His Ser Ser Pro Ala Ser Ser Asp Lys Asn Ser Asn Ser Asn
 1300 1305 1310
 His Arg Arg Arg Thr Ala Asp Ala Glu Ile Lys Met Gln Ser Met Gln
 1315 1320 1325
 Thr Pro Leu Gly Lys Thr Arg Ala Arg Ser Ser Gly Pro Thr Gln Val
 1330 1335 1340
 Pro Leu Pro Ser Ser Ser Phe Arg Ser Lys Gln Asn Val Lys Phe Ala
 1345 1350 1355 1360
 Ala Ser Val Lys Ser Lys Lys Pro Ser Ser Ser Ser Leu Arg Asn Ser
 1365 1370 1375
 Ser Pro Ile Arg Met Ala Lys Ile Thr His Val Glu Gly Lys Lys Pro
 1380 1385 1390
 Lys Ala Val Ala Lys Asn His Ser Ala Gln Leu Ser Ser Lys Thr Ser
 1395 1400 1405
 Arg Ser Leu His Val Arg Val Gln Lys Ser Lys Ala Val Leu Gln Ser
 1410 1415 1420
 Lys Ser Thr Leu Ala Ser Lys Lys Arg Thr Asp Arg Phe Asn Ile Lys
 1425 1430 1435 1440
 Ser Arg Glu Arg Ser Gly Gly Pro Val Thr Arg Ser Leu Gln Leu Ala
 1445 1450 1455
 Ala Ala Ala Asp Leu Ser Glu Asn Lys Arg Glu Asp Gly Ser Ala Lys
 1460 1465 1470
 Gln Glu Leu Lys Asp Phe Ser Tyr Ser Leu Arg Leu Ala Ser Arg Cys
 1475 1480 1485
 Ser Pro Pro Ala Ala Pro Tyr Ile Thr Arg Gln Tyr Arg Lys Val Lys
 1490 1495 1500
 Ala Pro Ala Ala Ala Gln Phe Gln Gly Pro Phe Phe Lys Glu
 1505 1510 1515

<210> 3
 <211> 1706
 <212> PRT
 <213> Rattus sp.

<400> 3

```

Met His Gln Asn Thr Glu Ser Val Ala Ala Thr Glu Thr Leu Ala Glu
 1           5           10           15
Val Pro Glu His Val Leu Arg Gly Leu Pro Glu Glu Val Arg Leu Phe
           20           25           30
Pro Ser Ala Val Asp Lys Thr Arg Ile Gly Val Trp Ala Thr Lys Pro
           35           40           45
Ile Leu Lys Gly Lys Lys Phe Gly Pro Phe Val Gly Asp Lys Lys Lys
           50           55           60
Arg Ser Gln Val Arg Asn Asn Val Tyr Met Trp Glu Val Tyr Tyr Pro
65           70           75           80
Asn Leu Gly Trp Met Cys Ile Asp Ala Thr Asp Pro Glu Lys Gly Asn
           85           90           95
Trp Leu Arg Tyr Val Asn Trp Ala Cys Ser Gly Glu Glu Gln Asn Leu
           100          105          110
Phe Pro Leu Glu Ile Asn Arg Ala Ile Tyr Tyr Lys Thr Leu Lys Pro
           115          120          125
Ile Ala Pro Gly Glu Glu Leu Leu Val Trp Tyr Asn Gly Glu Asp Asn
           130          135          140
Pro Glu Ile Ala Ala Ala Ile Glu Glu Glu Arg Ala Ser Ala Arg Ser
145          150          155          160
Lys Arg Ser Ser Pro Lys Ser Arg Arg Gly Lys Lys Lys Ser His Glu
           165          170          175
Asn Lys Asn Lys Gly Ile Arg Thr His Pro Thr Gln Leu Lys Ala Ser
           180          185          190
Glu Leu Asp Ser Thr Phe Ala Asn Met Arg Gly Ser Ala Glu Gly Pro
           195          200          205
Lys Glu Glu Asp Glu Arg Pro Leu Ala Ser Ala Pro Glu Gln Pro Ala
           210          215          220
Pro Leu Pro Glu Val Gly Asn Gln Asp Ala Val Pro Gln Val Ala Ile
225          230          235          240
Pro Leu Pro Ala Cys Glu Pro Gln Pro Glu Val Asp Gly Lys Gln Glu
           245          250          255
Val Thr Asp Cys Glu Val Asn Asp Val Glu Glu Glu Glu Leu Glu Glu
           260          265          270
Glu Glu Glu Leu Glu Glu Glu Glu Glu Glu Leu Gly Glu Asp Gly
           275          280          285
Val Glu Glu Ala Asp Met Pro Asn Glu Ser Ser Ala Lys Glu Pro Glu
           290          295          300
Ile Arg Cys Glu Glu Lys Pro Glu Asp Leu Leu Glu Glu Pro Gln Ser
305          310          315          320
Met Ser Asn Glu Ala Arg Glu Asp Ser Pro Asp Val Thr Pro Pro Pro
           325          330          335
His Thr Pro Arg Ala Arg Glu Glu Ala Asn Gly Asp Val Leu Glu Thr
           340          345          350
Phe Met Phe Pro Cys Gln His Cys Glu Arg Lys Phe Ala Thr Lys Gln
           355          360          365
Gly Leu Glu Arg His Met His Ile His Ile Ser Thr Ile Asn His Ala
370          375          380

```

Phe	Lys	Cys	Lys	Tyr	Cys	Gly	Lys	Arg	Phe	Gly	Thr	Gln	Ile	Asn	Arg	
385					390					395					400	
Arg	Arg	His	Glu	Arg	Arg	His	Glu	Thr	Gly	Leu	Lys	Arg	Arg	Pro	Ser	
				405					410					415		
Met	Thr	Leu	Gln	Ser	Ser	Glu	Asp	Pro	Asp	Asp	Gly	Lys	Gly	Glu	Asn	
			420					425					430			
Val	Thr	Ser	Lys	Asp	Glu	Ser	Ser	Pro	Pro	Gln	Leu	Gly	Gln	Asp	Cys	
		435					440					445				
Leu	Ile	Leu	Asn	Ser	Glu	Lys	Thr	Ser	Gln	Glu	Val	Leu	Asn	Ser	Ser	
	450					455					460					
Phe	Val	Glu	Glu	Asn	Gly	Glu	Val	Lys	Glu	Leu	His	Pro	Cys	Lys	Tyr	
465				470						475					480	
Cys	Lys	Lys	Val	Phe	Gly	Thr	His	Thr	Asn	Met	Arg	Arg	His	Gln	Arg	
				485					490					495		
Arg	Val	His	Glu	Arg	His	Leu	Ile	Pro	Lys	Gly	Val	Arg	Arg	Lys	Gly	
			500					505					510			
Gly	Leu	Leu	Glu	Glu	Pro	Gln	Pro	Pro	Ala	Glu	Gln	Ala	Pro	Pro	Ser	
		515					520					525				
Gln	Asn	Val	Tyr	Val	Pro	Ser	Thr	Glu	Pro	Glu	Glu	Gly	Glu	Thr		
	530					535					540					
Asp	Asp	Val	Tyr	Ile	Met	Asp	Ile	Ser	Ser	Asn	Ile	Ser	Glu	Asn	Leu	
545					550					555					560	
Asn	Tyr	Tyr	Ile	Asp	Gly	Lys	Ile	Gln	Thr	Asn	Ser	Ser	Thr	Ser	Asn	
			565						570					575		
Cys	Asp	Val	Ile	Glu	Met	Glu	Ser	Asn	Ser	Ala	His	Leu	Tyr	Gly	Ile	
			580					585					590			
Asp	Cys	Leu	Leu	Thr	Pro	Val	Thr	Val	Glu	Ile	Thr	Gln	Asn	Ile	Lys	
		595					600					605				
Ser	Thr	Gln	Val	Ser	Val	Thr	Asp	Asp	Leu	Leu	Lys	Asp	Ser	Pro	Ser	
	610					615					620					
Ser	Thr	Asn	Cys	Glu	Ser	Lys	Lys	Arg	Arg	Thr	Ala	Ser	Pro	Pro	Val	
625					630					635					640	
Leu	Pro	Lys	Ile	Lys	Thr	Glu	Thr	Glu	Ser	Asp	Ser	Thr	Ala	Pro	Ser	
			645						650					655		
Cys	Ser	Leu	Ser	Leu	Pro	Leu	Ser	Ile	Ser	Thr	Ala	Glu	Val	Val	Ser	
		660					665					670				
Phe	His	Lys	Glu	Lys	Gly	Val	Tyr	Leu	Ser	Ser	Lys	Leu	Lys	Gln	Leu	
		675					680					685				
Leu	Gln	Thr	Gln	Asp	Lys	Leu	Thr	Leu	Pro	Ala	Gly	Phe	Ser	Ala	Ala	
	690					695					700					
Glu	Ile	Pro	Lys	Leu	Gly	Pro	Val	Cys	Ala	Ser	Ala	Pro	Ala	Ser	Met	
705					710					715					720	
Leu	Pro	Val	Thr	Ser	Ser	Arg	Phe	Lys	Arg	Arg	Thr	Ser	Ser	Pro	Pro	
			725						730					735		
Ser	Ser	Pro	Gln	His	Ser	Pro	Ala	Leu	Arg	Asp	Phe	Gly	Lys	Pro	Asn	
			740					745					750			
Asp	Gly	Lys	Ala	Ala	Trp	Thr	Asp	Thr	Val	Leu	Thr	Ser	Lys	Lys	Pro	
		755					760					765				
Lys	Leu	Glu	Ser	Arg	Ser	Asp	Ser	Pro	Ala	Trp	Ser	Leu	Ser	Gly	Arg	
	770					775					780					
Asp	Glu	Arg	Glu	Thr	Gly	Ser	Pro	Pro	Cys	Phe	Asp	Glu	Tyr	Lys	Ile	
785					790					795					800	
Ser	Lys	Glu	Trp	Ala	Ala	Ser	Ser	Thr	Phe	Ser	Ser	Val	Cys	Asn	Gln	
			805						810					815		
Gln	Pro	Leu	Asp	Leu	Ser	Ser	Gly	Val	Lys	Gln	Lys	Ser	Glu	Gly	Thr	

820					825					830						
Gly	Lys	Thr	Pro	Val	Pro	Trp	Glu	Ser	Val	Leu	Asp	Leu	Ser	Val	His	
835					840					845						
Lys	Lys	Pro	Cys	Asp	Ser	Glu	Gly	Lys	Glu	Phe	Lys	Glu	Asn	His	Leu	
850					855					860						
Ala	Gln	Pro	Ala	Ala	Lys	Lys	Lys	Lys	Pro	Thr	Thr	Cys	Met	Leu	Gln	
865					870					875					880	
Lys	Val	Leu	Leu	Asn	Glu	Tyr	Asn	Gly	Val	Ser	Leu	Pro	Thr	Glu	Thr	
885					890					895						
Thr	Pro	Glu	Val	Thr	Arg	Ser	Pro	Ser	Pro	Cys	Lys	Ser	Pro	Asp	Thr	
900					905					910						
Gln	Pro	Asp	Pro	Glu	Leu	Gly	Pro	Asp	Ser	Ser	Cys	Ser	Val	Pro	Thr	
915					920					925						
Ala	Glu	Ser	Pro	Pro	Glu	Val	Val	Gly	Pro	Ser	Ser	Pro	Pro	Leu	Gln	
930					935					940						
Thr	Ala	Ser	Leu	Ser	Ser	Gly	Gln	Leu	Pro	Pro	Leu	Leu	Thr	Pro	Thr	
945					950					955					960	
Glu	Pro	Ser	Ser	Pro	Pro	Pro	Cys	Pro	Pro	Val	Leu	Thr	Val	Ala	Thr	
965					970					975						
Pro	Pro	Pro	Pro	Leu	Leu	Pro	Thr	Val	Pro	Leu	Ser	His	Pro	Ser	Ser	
980					985					990						
Asp	Ala	Ser	Pro	Gln	Gln	Cys	Pro	Ser	Pro	Phe	Ser	Asn	Thr	Thr	Ala	
995					1000					1005						
Gln	Ser	Pro	Leu	Pro	Ile	Leu	Ser	Pro	Thr	Val	Ser	Pro	Ser	Pro	Ser	
1010					1015					1020						
Pro	Ile	Pro	Pro	Val	Glu	Pro	Leu	Met	Ser	Ala	Ala	Ser	Pro	Gly	Pro	
1025					1030					1035					1040	
Pro	Thr	Leu	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Phe	Pro	Ser	
1045					1050					1055						
Ser	Cys	Ser	Ser	Thr	Ser	Pro	Ser	Pro	Pro	Pro	Leu	Ser	Ala	Val	Ser	
1060					1065					1070						
Ser	Val	Val	Ser	Ser	Gly	Asp	Asn	Leu	Glu	Ala	Ser	Leu	Pro	Ala	Val	
1075					1080					1085						
Thr	Phe	Lys	Gln	Glu	Glu	Ser	Glu	Ser	Glu	Gly	Leu	Lys	Pro	Lys	Glu	
1090					1095					1100						
Glu	Ala	Pro	Pro	Ala	Gly	Gly	Gln	Ser	Val	Val	Gln	Glu	Thr	Phe	Ser	
1105					1110					1115					1120	
Lys	Asn	Phe	Ile	Cys	Asn	Val	Cys	Glu	Ser	Pro	Phe	Leu	Ser	Ile	Lys	
1125					1130					1135						
Asp	Leu	Thr	Lys	His	Leu	Ser	Val	His	Ala	Glu	Glu	Trp	Pro	Phe	Lys	
1140					1145					1150						
Cys	Glu	Phe	Cys	Val	Gln	Leu	Phe	Lys	Val	Lys	Thr	Asp	Leu	Ser	Glu	
1155					1160					1165						
His	Arg	Phe	Leu	Leu	His	Gly	Val	Gly	Asn	Ile	Phe	Val	Cys	Ser	Val	
1170					1175					1180						
Cys	Lys	Lys	Glu	Phe	Ala	Phe	Leu	Cys	Asn	Leu	Gln	Gln	His	Gln	Arg	
1185					1190					1195					1200	
Asp	Leu	His	Pro	Asp	Glu	Val	Cys	Thr	His	His	Glu	Phe	Glu	Ser	Gly	
1205					1210					1215						
Thr	Leu	Arg	Pro	Gln	Asn	Phe	Thr	Asp	Pro	Ser	Lys	Ala	Asn	Val	Glu	
1220					1225					1230						
His	Met	Pro	Ser	Leu	Pro	Glu	Glu	Pro	Leu	Glu	Thr	Ser	Arg	Glu	Glu	
1235					1240					1245						
Glu	Leu	Asn	Asp	Ser	Ser	Glu	Glu	Leu	Tyr	Thr	Thr	Ile	Lys	Ile	Met	
1250					1255					1260						

Ala Ser Gly Ile Lys Thr Lys Asp Pro Asp Val Arg Leu Gly Leu Asn
 1265 1270 1275 1280
 Gln His Tyr Pro Ser Phe Lys Pro Pro Phe Gln Tyr His His Arg
 1285 1290 1295
 Asn Pro Met Gly Ile Gly Val Thr Ala Thr Asn Phe Thr Thr His Asn
 1300 1305 1310
 Ile Pro Gln Thr Phe Thr Thr Ala Ile Arg Cys Thr Lys Cys Gly Lys
 1315 1320 1325
 Gly Val Asp Asn Met Pro Glu Leu His Lys His Ile Leu Ala Cys Ala
 1330 1335 1340
 Ser Ala Ser Asp Lys Lys Arg Tyr Thr Pro Lys Lys Asn Pro Val Pro
 1345 1350 1355 1360
 Leu Lys Gln Thr Val Gln Pro Lys Asn Gly Val Val Val Leu Asp Asn
 1365 1370 1375
 Ser Gly Lys Asn Ala Phe Arg Arg Met Gly Gln Pro Lys Arg Leu Ser
 1380 1385 1390
 Phe Asn Val Glu Leu Gly Lys Met Ser Pro Asn Lys Leu Lys Leu Ser
 1395 1400 1405
 Ala Leu Lys Lys Lys Asn Gln Leu Val Gln Lys Ala Ile Leu Gln Lys
 1410 1415 1420
 Asn Arg Ala Ala Lys Gln Lys Ala Asp Leu Arg Asp Thr Ser Glu Ala
 1425 1430 1435 1440
 Ser Ser His Ile Cys Pro Tyr Cys Asp Arg Glu Phe Thr Tyr Ile Gly
 1445 1450 1455
 Ser Leu Asn Lys His Ala Ala Phe Ser Cys Pro Lys Lys Pro Leu Ser
 1460 1465 1470
 Pro Ser Lys Arg Lys Val Ser His Ser Ser Lys Lys Gly Gly His Ala
 1475 1480 1485
 Ser Ser Ser Ser Ser Asp Arg Asn Ser Ser Cys His Pro Arg Arg Arg
 1490 1495 1500
 Thr Ala Asp Thr Glu Ile Lys Met Gln Ser Thr Gln Ala Pro Leu Gly
 1505 1510 1515 1520
 Lys Thr Arg Ala Arg Ser Thr Gly Pro Ala Gln Ala Ser Leu Pro Ser
 1525 1530 1535
 Ser Ser Phe Arg Ser Arg Gln Asn Val Lys Phe Ala Ala Ser Val Lys
 1540 1545 1550
 Ser Lys Lys Ala Ser Ser Ser Ser Leu Arg Asn Ser Ser Pro Ile Arg
 1555 1560 1565
 Met Ala Lys Ile Thr His Val Glu Gly Lys Lys Pro Lys Ala Val Ala
 1570 1575 1580
 Lys Ser His Ser Ala Gln Leu Ser Ser Lys Ser Ser Arg Gly Leu His
 1585 1590 1595 1600
 Val Arg Val Gln Lys Ser Lys Ala Val Ile Gln Ser Lys Thr Ala Leu
 1605 1610 1615
 Ala Ser Lys Arg Arg Thr Asp Arg Phe Ile Val Lys Ser Arg Glu Arg
 1620 1625 1630
 Ser Gly Gly Pro Ile Thr Arg Ser Leu Gln Leu Ala Ala Ala Asp
 1635 1640 1645
 Leu Ser Glu Ser Arg Arg Glu Asp Ser Ser Ala Arg His Glu Leu Lys
 1650 1655 1660
 Asp Phe Ser Tyr Ser Leu Arg Leu Ala Ser Arg Cys Gly Ser Ser Thr
 1665 1670 1675 1680
 Ala Ser Tyr Ile Thr Arg Gln Cys Arg Lys Val Lys Ala Ala Ala Ala
 1685 1690 1695
 Thr Pro Phe Gln Gly Pro Phe Leu Lys Glu

1700

1705

<210> 4
 <211> 1506
 <212> PRT
 <213> Rattus sp.

<400> 4
 Met Arg Gly Ser Ala Glu Gly Pro Lys Glu Glu Asp Glu Arg Pro Leu
 1 5 10 15
 Ala Ser Ala Pro Glu Gln Pro Ala Pro Leu Pro Glu Val Gly Asn Gln
 20 25 30
 Asp Ala Val Pro Gln Val Ala Ile Pro Leu Pro Ala Cys Glu Pro Gln
 35 40 45
 Pro Glu Val Asp Gly Lys Gln Glu Val Thr Asp Cys Glu Val Asn Asp
 50 55 60
 Val Glu Glu Glu Glu Leu Glu Glu Glu Glu Glu Leu Glu Glu Glu
 65 70 75 80
 Glu Glu Glu Leu Gly Glu Asp Gly Val Glu Glu Ala Asp Met Pro Asn
 85 90 95
 Glu Ser Ser Ala Lys Glu Pro Glu Ile Arg Cys Glu Glu Lys Pro Glu
 100 105 110
 Asp Leu Leu Glu Glu Pro Gln Ser Met Ser Asn Glu Ala Arg Glu Asp
 115 120 125
 Ser Pro Asp Val Thr Pro Pro Pro His Thr Pro Arg Ala Arg Glu Glu
 130 135 140
 Ala Asn Gly Asp Val Leu Glu Thr Phe Met Phe Pro Cys Gln His Cys
 145 150 155 160
 Glu Arg Lys Phe Ala Thr Lys Gln Gly Leu Glu Arg His Met His Ile
 165 170 175
 His Ile Ser Thr Ile Asn His Ala Phe Lys Cys Lys Tyr Cys Gly Lys
 180 185 190
 Arg Phe Gly Thr Gln Ile Asn Arg Arg Arg His Glu Arg Arg His Glu
 195 200 205
 Thr Gly Leu Lys Arg Arg Pro Ser Met Thr Leu Gln Ser Ser Glu Asp
 210 215 220
 Pro Asp Asp Gly Lys Gly Glu Asn Val Thr Ser Lys Asp Glu Ser Ser
 225 230 235 240
 Pro Pro Gln Leu Gly Gln Asp Cys Leu Ile Leu Asn Ser Glu Lys Thr
 245 250 255
 Ser Gln Glu Val Leu Asn Ser Ser Phe Val Glu Glu Asn Gly Glu Val
 260 265 270
 Lys Glu Leu His Pro Cys Lys Tyr Cys Lys Lys Val Phe Gly Thr His
 275 280 285
 Thr Asn Met Arg Arg His Gln Arg Arg Val His Glu Arg His Leu Ile
 290 295 300
 Pro Lys Gly Val Arg Arg Lys Gly Gly Leu Leu Glu Glu Pro Gln Pro
 305 310 315 320
 Pro Ala Glu Gln Ala Pro Pro Ser Gln Asn Val Tyr Val Pro Ser Thr
 325 330 335
 Glu Pro Glu Glu Glu Gly Glu Thr Asp Asp Val Tyr Ile Met Asp Ile
 340 345 350
 Ser Ser Asn Ile Ser Glu Asn Leu Asn Tyr Tyr Ile Asp Gly Lys Ile
 355 360 365

Gln Thr Asn Ser Ser Thr Ser Asn Cys Asp Val Ile Glu Met Glu Ser																			
370						375				380									
Asn Ser Ala His Leu Tyr Gly Ile Asp Cys Leu Leu Thr Pro Val Thr																			
385						390				395									400
Val Glu Ile Thr Gln Asn Ile Lys Ser Thr Gln Val Ser Val Thr Asp																			
				405						410									415
Asp Leu Leu Lys Asp Ser Pro Ser Ser Thr Asn Cys Glu Ser Lys Lys																			
				420						425									430
Arg Arg Thr Ala Ser Pro Pro Val Leu Pro Lys Ile Lys Thr Glu Thr																			
				435						440									445
Glu Ser Asp Ser Thr Ala Pro Ser Cys Ser Leu Ser Leu Pro Leu Ser																			
				450						455									460
Ile Ser Thr Ala Glu Val Val Ser Phe His Lys Glu Lys Gly Val Tyr																			
465						470				475									480
Leu Ser Ser Lys Leu Lys Gln Leu Leu Gln Thr Gln Asp Lys Leu Thr																			
				485						490									495
Leu Pro Ala Gly Phe Ser Ala Ala Glu Ile Pro Lys Leu Gly Pro Val																			
				500						505									510
Cys Ala Ser Ala Pro Ala Ser Met Leu Pro Val Thr Ser Ser Arg Phe																			
				515						520									525
Lys Arg Arg Thr Ser Ser Pro Ser Ser Pro Gln His Ser Pro Ala																			
				530						535									540
Leu Arg Asp Phe Gly Lys Pro Asn Asp Gly Lys Ala Ala Trp Thr Asp																			
545						550				555									560
Thr Val Leu Thr Ser Lys Lys Pro Lys Leu Glu Ser Arg Ser Asp Ser																			
				565						570									575
Pro Ala Trp Ser Leu Ser Gly Arg Asp Glu Arg Glu Thr Gly Ser Pro																			
				580						585									590
Pro Cys Phe Asp Glu Tyr Lys Ile Ser Lys Glu Trp Ala Ala Ser Ser																			
				595						600									605
Thr Phe Ser Ser Val Cys Asn Gln Gln Pro Leu Asp Leu Ser Ser Gly																			
				610						615									620
Val Lys Gln Lys Ser Glu Gly Thr Gly Lys Thr Pro Val Pro Trp Glu																			
625						630				635									640
Ser Val Leu Asp Leu Ser Val His Lys Lys Pro Cys Asp Ser Glu Gly																			
				645						650									655
Lys Glu Phe Lys Glu Asn His Leu Ala Gln Pro Ala Ala Lys Lys Lys																			
				660						665									670
Lys Pro Thr Thr Cys Met Leu Gln Lys Val Leu Leu Asn Glu Tyr Asn																			
				675						680									685
Gly Val Ser Leu Pro Thr Glu Thr Thr Pro Glu Val Thr Arg Ser Pro																			
				690						695									700
Ser Pro Cys Lys Ser Pro Asp Thr Gln Pro Asp Pro Glu Leu Gly Pro																			
705						710				715									720
Asp Ser Ser Cys Ser Val Pro Thr Ala Glu Ser Pro Pro Glu Val Val																			
				725						730									735
Gly Pro Ser Ser Pro Pro Leu Gln Thr Ala Ser Leu Ser Ser Gly Gln																			
				740						745									750
Leu Pro Pro Leu Leu Thr Pro Thr Glu Pro Ser Ser Pro Pro Pro Cys																			
				755						760									765
Pro Pro Val Leu Thr Val Ala Thr Pro Pro Pro Pro Leu Leu Pro Thr																			
				770						775									780
Val Pro Leu Ser His Pro Ser Ser Asp Ala Ser Pro Gln Gln Cys Pro																			
785						790				795									800
Ser Pro Phe Ser Asn Thr Thr Ala Gln Ser Pro Leu Pro Ile Leu Ser																			

- 15 -

Asp Arg Glu Phe Thr Tyr Ile Gly Ser Leu Asn Lys His Ala Ala Phe
 1250 1255 1260
 Ser Cys Pro Lys Lys Pro Leu Ser Pro Ser Lys Arg Lys Val Ser His
 1265 1270 1275 1280
 Ser Ser Lys Lys Gly Gly His Ala Ser Ser Ser Ser Asp Arg Asn
 1285 1290 1295
 Ser Ser Cys His Pro Arg Arg Arg Thr Ala Asp Thr Glu Ile Lys Met
 1300 1305 1310
 Gln Ser Thr Gln Ala Pro Leu Gly Lys Thr Arg Ala Arg Ser Thr Gly
 1315 1320 1325
 Pro Ala Gln Ala Ser Leu Pro Ser Ser Ser Phe Arg Ser Arg Gln Asn
 1330 1335 1340
 Val Lys Phe Ala Ala Ser Val Lys Ser Lys Lys Ala Ser Ser Ser Ser
 1345 1350 1355 1360
 Leu Arg Asn Ser Ser Pro Ile Arg Met Ala Lys Ile Thr His Val Glu
 1365 1370 1375
 Gly Lys Lys Pro Lys Ala Val Ala Lys Ser His Ser Ala Gln Leu Ser
 1380 1385 1390
 Ser Lys Ser Ser Arg Gly Leu His Val Arg Val Gln Lys Ser Lys Ala
 1395 1400 1405
 Val Ile Gln Ser Lys Thr Ala Leu Ala Ser Lys Arg Arg Thr Asp Arg
 1410 1415 1420
 Phe Ile Val Lys Ser Arg Glu Arg Ser Gly Gly Pro Ile Thr Arg Ser
 1425 1430 1435 1440
 Leu Gln Leu Ala Ala Ala Ala Asp Leu Ser Glu Ser Arg Arg Glu Asp
 1445 1450 1455
 Ser Ser Ala Arg His Glu Leu Lys Asp Phe Ser Tyr Ser Leu Arg Leu
 1460 1465 1470
 Ala Ser Arg Cys Gly Ser Ser Thr Ala Ser Tyr Ile Thr Arg Gln Cys
 1475 1480 1485
 Arg Lys Val Lys Ala Ala Ala Thr Pro Phe Gln Gly Pro Phe Leu
 1490 1495 1500
 Lys Glu
 1505

<210> 5
 <211> 21
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> primer

<400> 5
 gggttggtgg tggttattgg g

21

<210> 6
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> primer

<400> 6
caaaaaccgc cctgcgccac tccttacc

28

<210> 7
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 7
gtggtgggta ttgggcgacg gc

22

<210> 8
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 8
gctatttcgc cgaccccgac g

21

<210> 9
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 9
tggtgggttat tgggtgatgg t

21

<210> 10
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 10
actatttcac caaccccaag a

21

<210> 11
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 11
aaaccatgga tcagaacact actgag 26

<210> 12
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 12
ccgtaagctt catgcagagg tgaaatctgg c 31

<210> 13
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 13
cgagccagcg cccggagcta agcttaagcg gagctcccc 39

<210> 14
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 14
gatttattag aggaatgaaa aacaacttca gaag 34

<210> 15
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 15
cttctgaagt tgtttttcat tcctctaata aatc 34

<210> 16
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 16
ctcattcatc taagaaaggt gg

22

<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 17
tgattccagg tcacttcagg

20

<210> 18
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 18
gaagccaaag gcctctcatc

20

<210> 19
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 19
agactctggc tgaggtacc

19